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PROCEEDINGS OF THE SYMPOSIUM ON CURRENT CONCEPTS AND APPROACHES ON ANIMAL TEST ALTERNATIVES

Harry Salem

RESEARCH AND TECHNOLOGY DIRECTORATE

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Biological and medical research has historically been conducted primarily with animal experimentation. The advances in biological scientific knowledge and medicine have been dependent and resulted from these animal experiments, supplemented with in vitro studies. Over the last decade, advocates for animal rights, and the skyrocketing cost of animal experimentation, have intensified the search for more alternatives to animal biomedical research and testing. Although to replace animal research is a noble quest, realistically this may never be accomplished since the final proof will require testing in an integrated whole animal system. However, the scientific and animal activist communities, sensitive to all of these issues are advocating the adoption of the 3 Rs: (1) replacement of animals; (2) reduction in the number of animals; (3) refinement by getting more information from fewer animals and using animals lower on the phylogenetic tree. A fourth R is taking responsibility for implementing the 3 R approach. These proceedings describe the current state of the art in the 4 Rs. Alternative methods (in vitro, chemical, computer) are described for not only eye and skin, but also for inhalation, aquatic, environmental and noncarcinogenic endpoints. In addition, the regulatory status is discussed since this is a major prerequisite to validation and acceptance.

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SUMMARY

Toxicology has always been an essential part of the Edgewood Arsenal, now the Chemical Research, Development and Engineering Center. Its mission is to develop the pharmacological/toxicological data base prior to fielding an item, and to provide experimental data necessary to predict adverse effects on the soldier following exposure to known and potential new chemical agents, as well as to determine permissible exposure limits (PEL's). Toxicological data is essential for modelers who develop scenarios ranging from when troops should mask to casualty estimates. In addition, experimental animal studies are conducted to determine the effectiveness of protective equipment, clothing and decontaminating agents. In the development of safe smoke and obscurants and riot control agents, toxicological data is also required. Toxicological data on mammals, earthworms and aquatic organisms, environmental fate and effects data in soils and plants are provided for incorporation into environmental assessments and environmental impact statements.

Edgewood has a history of prominence in Toxicology since its establishment in 1917. Some of the principles of inhalation toxicology were actually developed here, and are still being used in the field. In the practice of toxicology at Edgewood we subscribe to the Code of Ethics of the Society of Toxicology, the Animal Welfare Act, the American Association for Accreditation of Laboratory Animal Care (AAALAC), and the Good Laboratory Practices.

Being sensitive to the use of experimental animals and the increased cost of mammalian research, we have committed to a program in alternatives to animal testing. This is now included in the mission statement of all of the Branches within Toxicology Division. We subscribe to the Three R's (Refinement, Reduction and Replacement), plus the Fourth R of Responsibility. Toxicology Division has been involved in the search and application of Alternatives since at least, the early 1980s, and have participated in many symposia and meetings on this subject.

Because of the diverse efforts ongoing in the Alternative Field, and the slow regulatory acceptance, we felt that it was important to sponsor a symposium, not only on eye irritation alternatives, but in all areas of

alternative research, to evaluate the progress to date, determine future direction, and understand the regulatory climate for using alternatives.

We are all committed to the use of alternatives (computer models, animals lower on the phylogenetic scale, tissue/cell cultures, chemical surrogates) where possible without compromising the safety and welfare of the soldier as well as the general public.

We have assembled leaders in the field to serve on the Steering and Local Committees, and have invited speakers who are actively making contributions to this field.

I thank all who have contributed and helped to bring this program to fruition, and trust that the symposium will be a participative and successfull one.

Harry Salem

SYMPOSIUM CHAIRMAN

HARRY SALEM, Ph.D.

Dr. Harry Salem is Chief of the Toxicology Division, Chemical Research, Development and Engineering Center, Aberdeen Proving Ground, MD. His research interests and experience are in inhalation and general toxicology and pharmacology. He has served on the editorial boards of several professional journals, and is the editor-in-chief of the Journal of Applied Toxicology. He has served as an associate professor of pharmacology at the University of Pennsylvania, and adjunct professor of environmental health at Temple University. He was also employed by small and large industry in pharmacology and toxicology. He is chairman of the Technical Committee for the Specialty Section on Inhalation of the Society of Toxicology. His professional affiliations include: American Chemical Society, American College of Toxicology, the American Society for Pharmacology and Experimental Therapeutics, the International Society of Regulatory Toxicology and Pharmacology, the International Society on Toxicology, Sigma Xi, and serves on the Steering Committee for the Society of Comparative Opthalmology. He is a Fellow of the New York Academy of Sciences, the American College of Clinical Pharmacology, and of the Academy of Toxicological Sciences, where he served on the Professional Standards Evaluation Board. He has contributed many scientific papers and is the co-editor of three volumes of the International Encyclopedia of Pharmacology and Therapeutics on Antitussive Agents and editor of the text, "Inhalation Toxicology."

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PREFACE

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Acknowledgments

These proceedings are dedicated to Flo, Jerry, Amy, and Joel for their practice of compassion and respect for all living things, and to all of the investigators who are seeking alternatives to animal testing without compromising human and animal health and welfare.

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CONTENTS

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PROCEEDINGS OF THE SYMPOSIUM ON CURRENT CONCEPTS AND APPROACHES ON ANIMAL TEST ALTERNATIVES

INTRODUCTION - ALTERNATIVE SYMPOSIUM

HARRY SALEM

This symposium has been organized by members of the scientific community who are sensitive to the issues of animal experimentation as well as to human health concerns and who are searching to provide validated substitutions for in-vivo animal models.

The objectives of this symposium are to present new concepts and approaches on alternatives to animal testing, as well as to review their stage of development and validation. We will, over the next few days focus on the Three R's, Refinement, Reduction and Replacement.

At the present time, tests in intact animals are the primary means of assessing potential hazards of chemical exposure in man, other than testing in man. Until such time as we can completely and confidently replace animals in testing by valid and meaningful models, we should attempt to obtain more information fr n each animal, which constitutes Refinement, and reduce the number of animals in an expeditious manner.

Let us review some of the reasons why we do animal experimentation and testing. Historically, laws were passed in response, retrospectively, to catastrophic events which impacted on the health and safety of the American people. The first Pure Food and Drug Act passed in 1906 resulted from adulteration of foods with chemical preservatives, "quack" remedies being promoted and sold to the consumer, and filth and contamination in the meat packing industry, conditions which were exposed in Upton Sinclair's book,"The Jungle". This Act also known as the Wiley Act, prohibited the manufacture and interstate shipment of adulterated and misbranded foods and drugs. The next major relevant law passed, was the Federal Food, Drug and Cosmetic Act of 1938. This was prompted, in part, by the Elixir of Sulfanilamide episode that resulted in over 100 deaths. Although sulfanilamide tablets and powder had previously been used safely for the treatment of streptococcal infections, the elixir formulated with ethylene glycol as the vehicle had not been tested for toxicity, nor was it a requirement of law up till that time. This new law of 1938, also regulated cosmetics. As a result, the Draize Eye Irritation Test was developed and used to prevent dangerous products such as Lash-Lure a mascara which caused permanent blindness in a woman after its use in the early 1930's. A subsequent catastrophic event was that of Thalidamide which caused birth defects when it was taken

during pregnancy. This event resulted in the 1962 Kefauver-Harris Amendment to the Food, Drug and Cosmetic Act which added the requirement for additional animal testing not only for safety, but also for efficacy.

Today and in the future, with our advancing knowledge and technology, more testing may be mandated in order to rule out long term adverse effects, other than cancer, following long term exposure. Some of these potential adverse effects may even be unknown to us today.

Hopefully, much of the testing may be done adequately in-vitro, but ultimately the testing will require whole animal studies to understand the complex, dynamic interactions and functions of defense and repair mechanisms programmed in the intact animal to maintain homeostasis, This concept of homeostasis was first postulated by Claude Bernard almost 150 years ago.

As we refine our methodology in whole animal studies, in concert with in-vitro tests, the number of animals required for final testing may be reduced and not compromise the generation of an adequate and useful data base to ensure efficacy and safety.

Animal research has contributed significantly to many medical breakthroughs as well as to our data base of biological knowledge.

Since 1901, fifty-four of the seventy-six Nobel Prizes awarded in Physiology and Medicine have been made for discoveries based on the use of animals. Dr. Joseph Murray, the winner of the 1990 Nobel Prize for Medicine has stated, "there would not be a single person alive today as a result of an organ transplant or bone marrow transplant without animal experimentation". There is much evidence that remarkable improvement of health and well being (quality of life) has been dependent on animal research. Diabetes, once a killing disease, took its toll with tragic rapidity till the summer of 1921, when two young Canadian scientists, Frederick Banting and Charles Best, succeeded in isolating insulin which now enables millions of diabetics to lead normal lives. It was in these same laboratories at the University of Toronto, where I did my graduate research, that Banting and Best kept the depancreatized dog, Marjorie, alive with injections of pancreatic extracts, which is what we now know as insulin.

The development and safety testing of vaccines including the vaccine that led to the eradication of polio, was also dependent on animal experimentation and safety testing. Animal research has resulted in many

medical advances from drug development for the treatment of life threatening allergies, psychiatric disorders, heart disease and infections to transplantation and transplant therapy as well as surgery, including open heart and coronary bypass procedures.

Recently when Dr. Steven Rosenberg of NIH received a Research and Development Achievement Award for the development of a most promising breakthrough in the treatment of cancer using immunotherapy, he stated that none of this would have been possible without the use of animals.

It is readily obvious, that animal experimentation has played a very important role in improving our health and well being. Can we continue to make significant health and safety advances as well as breakthroughs, without animal experimentation? This is among the reasons why we are gathered here for the next few days.

However, as long as animal experimentation continues, being responsible and sensitive scientists, we are concerned about the use, care and welfare of laboratory animals. As a scientific community, we have accepted and subscribe to rules and guideline within which we conduct our animal research. These include the American Association for the Accreditation of Laboratory Animal Care (AAALAC) Accreditation, Laboratory Animal Use Committee review, and compliance with the Animal Welfare Act, Code of Ethics of the Society of Toxicology, and Good Laboratory Practices.

We are all aware of the many advances in health and safety that have been accomplished through animal research. Is it possible to do the same without animal experimentation? Those of us who planned and organized this symposium, as well as the attendees here, are all dedicated to pursue this approach by being committed to the principles of the Three R's without compromising the health and safety of our population. We hope that by the use of in-vitro systems, such as cell lines, isolated cells, microorganisms, tissue cultures, invertebrates, quantitative structure activity relationships and physiologically based pharmacokinetic modeling, we will be able to predict toxicity or at least gain insight as to the mechanisms of action and possible target organs. These techniques should result in a reduction in animal experimentation.

During the next few days, we will discuss the methods available to us, their stage of development and validation, and how they

can be applied so that we can implement the Three R's in efficacy and safety testing of drugs and chemicals.

I look forward to your participation in making this a very worthwhile and productive meeting.

RELATING IN VITRO TO IN VIVO EXPOSURES WITH PHYSIOLOGICALLY-BASED TISSUE DOSIMETRY AND TISSUE RESPONSE MODELS

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Abstract: Physiologically-based models describe the interrelationships of processes at the molecular, biochemical, cellular and organ system levels that determine the delivery of chemicals to target tissues and the responses of tissues to the chemical. These models, which generally consist of sets of differential equations, encode various biological relationships in mathematical form and, in principle, permit extrapolation from high-to-low doses, from one species to another, and from one dose route to other dose routes. They also provide a method to extrapolate from results with in vitro systems to make predictions about expected behavior in vivo. This in vitro to in vivo extrapolation is accomplished by obtaining quantitative data on tissue solubilities/tissue binding, tissue-toxicant interactions, rates of metabolism, and concentration-response relationships in vitro and using this information in the physiological models to simulate expected outcome of exposure in the intact animal. These physiological models allows us to relate in vitro to in vivo exposure conditions. The more pervasive use of in vitro-in vivo extrapolation efforts will depend on successful demonstration of the accuracy of these extrapolations by comparing predictions from physiological dosimetry and response models that utilize parameters derived from in vitro studies with actual experimentation in vivo. This paper examines the integration of physiological modeling strategies with in vitro toxicity studies and the potential role of these integrated strategies in risk assessments.

Introduction: The assessment of potential health risks associated with human exposure to chemicals is performed in four steps: (1) hazard identification, (2) exposure assessment, (3) dose-response assessment, and (4) risk characterization (National Academy of Sciences, 1983). Toxicology studies in animals and in animal tissue preparations provide qualitative and quantitative information useful for risk assessment. Qualitative information is usually more useful for hazard identification, i.e., estimating what kinds of adverse responses may be caused by a particular chemical. Quantitative results provide information for the dose-response assessment, i.e., deciding under which exposure conditions these effects are likely to occur in animals and in exposed people. In vitro systems are often applied to study (1) metabolic rates and pathways of metabolism, (2) mechanisms of action of the toxicant and (3) tissue responses, using tissue from various species including humans. These in vitro test systems have been more frequently applied for hazard identification than for dose-response assessment, even though these systems are amenable to detailed characterization of quantitative dose-response relationships.

To utilize this rich in vitro data source in the dose-response assessment portion of contemporary risk assessment, we need to know how the observations in vitro relate to in vivo exposure situations. Two important questions are: What types of in vivo exposures give rise to tissue and cellular concentrations equivalent to those achieved in the in vitro studies and, how accurately do in vitro dose-response curves predict dose-response behavior in the intact organism? Physiologically-based dosimetry and physiologically-based tissue response models permit a wide range of extrapolations (Clewell and Andersen, 1985; Leung, 1991), including extrapolation from results with multiple in vitro test systems to predict toxicity and

dose response characteristics under realistic exposure conditions in the living animal (Krishnan and Andersen, 1992).

The interested reader is directed to several reviews related to aspects of physiologically-based modeling (Gerlowski and Jain, 1983; Himmelstein and Lutz, 1979; D'Souza and Boxenbaum, 1988). This paper discusses the integration of in vitro results via these physiologically-based models and the prospects for wider application of these integrated techniques in chemical risk assessment, including extrapolation based on in vitro results with human tissue samples.

Integrated Exposure-Dose-Response Relationships: Toxicity in intact animals represents the culmination of a series of interrelated steps; exposure, delivery of active chemical from the external environment to target tissues, initial interaction of toxic chemical with cellular constituents, and progression from these initial interactions to cellular alterations and eventually to overt deleterious responses at the cellular, tissue or organism level. Schematically,

EXPOSURE ---> TARGET TISSUE DOSE ---> INITIAL CELLULAR INTERACTIONS

and then,

INITIAL CELLULAR INTERACTIONS --> ALTERED CELL FUNCTION --> TOXICITY

Tissue dose of the toxic moiety, the unchanged parent chemical or reactive metabolites, is not always proportional to exposure concentration of the parent chemical, especially at the higher exposure levels frequently used in toxicological

studies (e.g., cancer bioassays). In these cases, tissue disposition of a chemical changes from one exposure level to another and the basis of this exposure-level dependent behavior has to be ascertained to adequately predict response incidence across exposure levels. To distinguish the concepts of exposure level and dose to critical tissues, the use of the term 'exposure-dose-response assessment' has been proposed (Andersen et al., 1992). The expression, exposure-dose-response assessment, refers to the quantitative relationship between exposure levels and target tissue dose, and further the relationship between tissue dose and the observed/predicted responses in animals and people.

In vitro toxicology studies typically produce concentration-response relationships with respect to particular endpoints. Ideally, the goal of these in vitro studies should be to predict accurately the exposure-dose-response relationship for the intact animal. To date, little attention has been paid to the ability of these in vitro results to quantitatively predict outcome in vivo. Progress in relating the exposures and toxicity observed in vitro to in vivo conditions has been hampered by lack of application of quantitative tools. Understanding the critical biological determinants that constitute the in vitro and in vivo systems should make it feasible to determine the in vivo conditions that produce tissue concentrations and toxic effects comparable to those seen in vitro. This comparison can be effectively accomplished when in vitro data are incorporated within a physiologically-based modeling framework.

In vitro and in vivo exposures: The concentration-response relationship obtained in vitro may well be observed in the intact test organism provided (1) the exposure concentrations are comparable and (2) the critical tissue response/repair elements are adequately expressed in the in vitro system. The exposure concentration in in

vitro experiments refers to the concentration of chemical in the incubation medium, i.e., in proximity to the target cells or tissues. In vitro concentrations can generally be easily estimated by appropriate chemical assays. The in vivo exposure concentration refers to the concentration in the ambient environment. To compare in vitro and in vivo concentrations, the concentration in proximity to target cells in vivo have to be compared to the in vitro exposure conditions. Concentrations, in this regard, may relate to parent chemical or reactive metabolite(s). In vitro and in vivo exposures would be considered similar when the appropriate tissue dose metric is found to be similar for the in vivo exposure and the in vitro test situation. To be of practical value then, we need to be able to determine or reliably estimate the external exposure conditions that would give rise in vivo to the instantaneous or cumulative tissue dose achieved by the in vitro exposure situation. The ability to compare these situations requires a quantitative description that relates tissue dose to exposure concentration within a pharmacokinetic framework. Physiologically-based pharmacokinetic models provide this framework.

Physiological Dosimetry Models: The steps from exposure to tissue dose to initial cellular effects are frequently included in descriptions of the pharmacokinetics or dosimetry of toxic chemicals. The accumulation and elimination of precursor chemical and reactive metabolites from each tissue within a physiologically based pharmacokinetic (PB-PK) model (Fig 1) are described by appropriate mass-balance differential equations (MBDE). Terms and abbreviations used frequently in the equations are given in Table 1. A representative MBDE for the liver, expressing the rate of change of the amount of chemical in the liver (A_l), usually contains terms for parent chemical entering and exiting the tissue via the blood flow (Q_l) with arterial (C_a) and venous blood

(Cvl) concentrations and for loss (or possibly gain) of chemical in the tissue due to metabolism. The MBDE for the liver below-Equation (1) - includes two pathways of metabolism, a saturable component and a first-order component.

$$dA_l/dt = Q_l * (C_a - C_{vl}) - V_{max} * C_{vl} / (Km + Cvl) - k_f * C_{vl} * V_l \qquad (1)$$

In most PB-PK models, amount of chemical in the tissue (A_t) is determined by integrating equation (1) from an appropriate starting condition ($A_{l,O}$) by numerical techniques.

$$A_l = \text{integral}\ (\ dA_l / dt) + A_{l,0} \qquad (2)$$

The concentration in the tissue (C_t) is estimated by dividing the amount in the tissue (A_t) by the tissue volume (V_t); the free concentration, in well-mixed, flow-limited model structures (Andersen, 1991), is the tissue concentration divided by the tissue blood partition coefficient (P_t). Thus, for the liver,

$$C_l = A_l / Vl \qquad (3) \quad \text{and} \quad C_{vl} = C_l / P_l \qquad (4)$$

The full model contains a MBDE for each tissue.

In other cases, such as methotrexate binding to dihydrofolate reductase (Bischoff et al., 1971), or dioxin binding to cytochrome P450-1A2 in the liver (Leung et al., 1990), there may be specific tissue binding interactions. Now, the mass conservation equation contains free, partitioned and specifically bound forms of the chemical.

$$A_t = C_{vt}*P_t*V_t + B_{max}*C_{vt}/(K_b + C_{vt}) \qquad (5)$$

Metabolism of certain xenobiotics produces metabolites that can react with cellular constituents to elicit toxic effects. The rate of change of the amount of reactive metabolites (A_{rm}) available for reaction with critical macromolecules (MM) in a tissue depends on the rate of formation in the tissue, the rate of reaction with the target macromolecule(s), and the rate of detoxifying reactions in the tissue.

$$dA_{rm}/dt = \text{formation - reaction with target molecules - detoxification}$$

For the case where the saturable metabolism of parent chemical produces a reactive intermediate, the MBDE for the amount of reactive metabolite (A_{rm}) and the steady-state concentration of the reactive metabolite ($C_{rm,ss}$) are:

$$dA_{rm}/dt = V_{max}*C_{vt}/(K_m + C_{vt}) - k_{mm}*V_t*C_{rm}*MM - k_{dt}*C_{rm}*V_t \qquad (6)$$

$$\text{and,} \quad C_{rm,ss} = (V_{max}*C_{vt}/(K_m + C_{vt}))/(k_{mm}*MM + k_{dt}*R) \qquad (7)$$

When these metabolites are highly reactive and never circulate beyond the tissue in which they are produced, these simple equations serve to predict the average target tissue exposure to C_{rm}. In other cases, the metabolites are reactive, but stable, and are eliminated by metabolism after distribution via the systemic circulation. The PB-dosimetry model then has to permit circulation and access of these metabolites to other tissues. Separate PB-models then are provided for the important metabolites (Deitz et al., 1981; Gargas et al., 1986; Fisher et al., 1991).

The next process to be included in these dosimetry models is the initial cellular interactions of parent chemical or reactive metabolite with cellular target molecules. These cellular interactions correspond to the necessary initiating steps linking tissue dose to subsequent toxicity. A few examples may be useful here. A reactive, mutagenic parent compound such as ethylene oxide, reacts with DNA to form DNA-adducts that, when present during cell division, increase the probability of mutation and malignant transformation (Krishnan et al., 1992). Other reactive species such as the trichloromethyl free radical formed by carbon tetrachloride metabolism react with proteins and lipids, altering the properties of these macromolecules (Recknagel, 1967). Sufficient degradation of the pool of functional macromolecule leads to cell damage and cell death. With dioxin, interactions between dioxin and a cellular protein, the *Ah* receptor, eventually lead to changes in cell concentrations of growth factors which appear, in a complex fashion, to alter cell growth and differentiation rates (Whitlock, 1990; Sutter et al., 1991).

The accumulation of protein or DNA adducts is expected to be related to the average tissue concentration of the reactive chemical species (either C_{vt} or C_{rm} or a combination of the two). The time average concentration of an adduct formed from a reactive metabolite is dependent on rates of formation and tissue repair of the adduct. The equation for the change in concentration of the adduct and the steady-state concentration can be expressed in a generic form, where formation is via a second order reaction and repair is first-order with respect to the adduct concentration.

$$d(\text{Adduct})/dt = k_{mm} * C_{rm} * MM - k_{repair} * (\text{Adduct}) \qquad (8)$$

and,

$$(\text{Adduct})_{ss} = k_{mm} * C_{rm} * MM / k_{repair} \qquad (9)$$

With dioxin (Whitlock, 1990) or peroxisomal proliferators (Green, 1991), the initial cellular interactions are believed to occur via specific protein receptors; the chemical and receptor form a complex which alters gene transcription. Protein induction by dioxin, for instance, has been modeled by assuming an interaction between dioxin, the *Ah* receptor and sites on DNA (Andersen et al., 1993). Dioxin modified the zero-order rate of synthesis (k_{syn}), but not the first order rate constant of degradation (k_{degrad}). Thus, the steady-state concentration of protein or growth factor becomes:

$$(\text{Growth Factor}_{ss}) = k_{syn} * (1 + (\text{L-}Ah\text{-DNA})^n / (K_d^n + (\text{L-}Ah\text{-DNA})^n) - k_{degrad} * (\text{Growth Factor}) \qquad (10)$$

where L-*Ah*-DNA is the concentration of dioxin-*Ah* receptor-DNA complexes within the cell. The relationship depends on the affinity of the dioxin-*Ah* complex for the sites on DNA, given by the dissociation constant K_d. The exponent, n, allows co-operativity in binding to multiple sites. These physiological models for initial cellular interactions, i.e., DNA binding, protein/lipid binding, and growth factor expression, bridge the gap between tissue dosimetry and tissue response models for specific toxic effects.

Physiological response models: These initial cellular interactions lead to an impact on cellular function and eventually to toxic effects. These response relationships can also be described by quantitative physiological modeling approaches and such relationships have been developed for a limited number of

endpoints (Conolly and Andersen, 1991). Probably, the most extensive work has focused on models for chemical carcinogenesis, that themselves derive from more general attempts to develop biologically realistic two-stage models of cancer (Armitage and Doll, 1954) that include mutation rates and growth rates of the normal and intermediate cell types involved in the progression to cancer (Moolgavkar, 1986).

In the two stage, Moolgavkar, Venzon, Knudson (MVK) cancer model, tumors arise from the sequential mutation of both alleles of a critical gene. The tumor incidence function depends on the number of susceptible cells, N_0, the mutation rate for conversion of normal cells to intermediate cells with one mutation, μ_1, the birth and death rates of these intermediate cells, α_2 and β_2, respectively, and the mutation rate for conversion of intermediate to fully malignant cells, μ_2. For time independent parameters, the approximate incidence at any time , I(t), is:

$$I = \mu_1 * \mu_2 * N_0 \int_0^t \exp\,(\alpha_2 - \beta_2)(t-s)\, ds \qquad (11)$$

The complete exposure-dose-response model for chemical carcinogenesis includes the necessary relationships between tissue dose, i.e. chemical concentrations, protein/DNA adducts, or growth factor concentrations, and the mutation, birth, and death rates in these cancer response models.

In creating dose-response models for an individual response, for instance, cell death (β_2), we generally lack sufficient detail to accurately define all the steps at the cellular level and are forced to define mathematical functions that correlate the response with adduct level or with growth factor level. One possible relationship is linearity between our measure of the initial cellular interactions and response.

A linear relationship may exist, for example, between mutation rates (μ_1,μ_2) and DNA-adduct levels or perhaps for the relationship between cell proliferation rates (α_2)and growth factor concentrations. More complex relationships have been suggested for rates of cell death, where the sensitivity to death has been expressed as a log-normal probability distribution of the amount of protein adducts or the rate of metabolism to reactive intermediates (Reitz et al, 1990; Bogen, 1990). Mutation, cell growth/differentiation rates, and cell death rates influence various toxicity end-points, including immunotoxicity, specific organ toxicity, and teratogenesis, in addition to cancer.

This brief summary outlines the general structure of a few PB-dosimetry and PB-tissue response models. There are of course a broad diversity of such models, with significant individualization required for specific chemicals (Gerlowski and Jain, 1983; D'Souza and Boxenbaum, 1988). The purpose of providing these examples and the equations that are contained in these models is to focus attention on the individual biological determinants that underlie these exposure-dose-response models and to begin to examine the areas in which in vitro studies can contribute to development of these models. The ultimate goal of the integration of physiological modeling strategies with in vitro results is to show that in vitro results, placed in the context of these predictive physiological models, generate predictions of dosimetry, initial cellular interactions, and toxicity that compare well with observations in test animals.

In vitro studies and tissue dosimetry: To apply these models to predict tissue dosimetry, it is necessary to have estimates of the physiological, biochemical, and biological parameters in these various equations. The anatomical and physiological parameters in a test species are usually available from the literature and require

little additional research studies on whole animals. Many of the biochemical and physio-chemical parameters can potentially be obtained from in vitro systems (Table 2). Among the important areas where in vitro studies contribute are skin or gut preparations to determine the rates, extent , and mechanisms of absorption of xenobiotics; tissue, cellular, and subcellular preparations to assess rates and affinities of metabolizing enzyme pathways; and various intact tissue preparations to assess the initial interactions between toxic chemicals and target tissues.

Chemicals gain entry to tissues via various routes of administration; inhalation, skin application, ingestion, etc., where the chemicals have to diffuse through cellular barriers before reaching the circulation. The use of appropriate in vitro models for absorption with skin and gut could permit characterization, on a quantitative level, of the determinants of absorption and inclusion of this information in the models (Hotchkiss et al., 1990; Dykes et al., 1991). Presently, the only way to accurately examine the rate of absorption with most chemicals is to expose the animals and measure blood concentrations at various times after administration.

In the body some clearance mechanisms remove chemical directly from the circulation. Organic acids, like penicillin, are removed by filtration and secretion into the kidney filtrate. Organic dyes are eliminated by biliary excretion after clearance from the hepatic blood flow. The kinetics of these chemicals depends on their solubilities in tissue and the blood flow to these specific organs. By far the most common mode of elimination of chemicals from the body is via metabolism. In vitro methods have been widely used to examine the nature of metabolites formed and rates of biotransformation of various toxic chemicals.

These studies have been performed with isolated perfused tissues, tissue slices, isolated cells from the target tissues, and various subcellular preparations, mitochondria, microsomes, etc. But do these in vitro preparations give results consistent with metabolic clearance in the living animal? Some limited work has focused on the quantitative evaluation of the ability of in vitro kinetic constants to predict clearances and half-lives in vivo. Examples include studies with drugs (Rane et al., 1976) and with volatile organic compounds of interest due to their presence in the general environment and in occupational settings (Hildebrand et al., 1981; Sato and Nakajima, 1979a). Frequently, the units employed by investigators using in vitro systems are not conducive to extrapolation. Maximum velocities are expressed per unit microsomal protein, with no indication of the yield of protein per unit liver weight. For PB-PK modeling the Vmax needs to be expressed per weight of tissue or per total tissue. When metabolic rates are also accurately known for reactions of metabolite(s), this information can be integrated to generate PB-PK models that describe the disposition of both parent chemical and major reaction products.

The amount of chemical in a tissue depends on the partitioning and tissue binding characteristic of the material. In vitro techniques have been used to estimate partition coefficients for volatile chemicals and partitioning of both charged and neutral, non-volatile chemicals (Gargas et al., 1988; Fiserova-Bergerova and Diaz, 1986; Sato and Nakajima, 1979b; Lin et al., 1982). Binding relationships with particular receptors are characterized in vitro with various tissue preparations and analysis with Scatchard plots, or by other suitable techniques. These constants together with blood flow rates and rates of metabolism when integrated in Equations (1, 5) permit estimation of tissue concentrations of these test chemicals. Non-linear processes are accommodated by capacity limited binding (B_{max}) and

metabolism (V_{max}), or sometimes by changes in physiological parameters, such as altered body composition during aging (Birnbaum, 1991), or by pharmacological effects of exposure on blood flows or breathing rates.

Comparing Exposures: In vitro studies sometimes precede detailed in vivo toxicity research. A question that always lurks near the surface when discussing the results of these in vitro toxicity results is whether the concentrations used have any relevance at all to in vivo exposure conditions. It is frequently possible to expose cells in suspension or tissues in culture to concentrations of chemical that would be impossible to achieve, even under the most unrealistic exposure situations. How do we know when this over-exposure in vitro is a problem for interpretation? One possibility involves the application of these PB-dosimetry models. These predictive PB-PK models readily serve as tools to assess the relevance of these in vitro exposure concentrations. In these cases, PB-PK models estimate the in vivo exposure conditions necessary to produce the concentrations achieved in vitro. If the conclusion is reached that these tissue concentrations only arise in the animal at completely unreasonable exposure levels, the in vitro system should be regarded to be of little or no relevance for predicting human risk.

The reverse application is equally possible. Once all necessary model parameters are known, these models can predict target tissue concentrations of chemical and metabolite achieved during those in vivo exposures that give rise to overt effects in the animal. These concentrations can then be used as the test concentrations for studies with in vitro cell systems. In vitro concentrations probably should not be used if they exceed overtly toxic concentrations in vivo by more than several-fold.

Tissue response modeling: The initial interactions of chemicals and reactive metabolites with tissues are easily evaluated in any number of in vitro preparations. Hepatocytes, renal tubule cells, purified lung clara cells, among a host of similar tissue preparations, are useful for evaluating specific tissue metabolism, including activation of chemicals to metabolites that react with target macromolecules in the cell. Repair processes for clearance of adducts could be studied in isolated cells in culture, for instance. The elaboration of specific growth factors could also be evaluated in appropriate cell lines or tissue preparations and correlated with cell proliferation or cell differentiation rates. Many other end points could also be evaluated, with judicious choice of cell system and exposure conditions. One longer-term advantage of work with cell systems is the possibility of conducting similar work on human tissue as appropriate. Human hepatocytes could be used side by side with animal hepatocytes to estimate rates of metabolism, extent of macromolecular binding, and sensitivity to damage. When the in vitro - in vivo extrapolation procedures for rats were sufficiently well-developed, they could readily be applied to the results from human tissues. Successful in vitro to in vivo extrapolation could reduce our reliance on animal test for estimating expected human health risks. Instead of relying on extrapolations from animal test results, in vitro toxicity tests with human tissues could be used together with physiological modeling to conduct dose-response evaluations. Animal studies, of course, would still be a mainstay of hazard identification studies, along with appropriate in vitro response studies.

Dose-response relationships: These in vitro cell and tissue systems bridge the gap between dosimetry and tissue response at the level of the intact animal. Some examples for our three cases discussed above include in vitro hepatotoxicity of chlorinated aliphatic chemicals (Tyson, 1987; Tyson et al., 1989), in vitro analysis

of DNA repair and replication (Doolittle et al., 1987), and in vitro correlation between DNA adduct formation and chromosomal breakage (Walk et al., 1989). These systems also begin to permit examination of the quantitative dose-response behavior relating initial cellular interactions and toxicity. The dose response relationships give the information on the intensity of response at different dose levels. For instance, they describe the probability of mutation arising due to a particular concentration of DNA-adducts in a cell, or the probability of cells dying due to a particular concentration of macromolecular adducts in the target cells, or the probability of cell proliferation due to a particular increase in growth factor concentration. Integrating these in vitro dose-response curves with the PB-PK models permits prediction of the dose-response behavior in the intact animal.

While the in vitro-in vivo extrapolation of dosimetry and some of these initial cellular interactions appears very possible, more advanced pathological states undoubtedly represent the interplay of complex biological processes mediated by multiple organ systems in the body and may be very difficult to predict based simply on in vitro results from cells from a single tissue examined in vitro. Cell death may follow macromolecular binding, but aspects of cell-cell communication in the intact tissue or elaboration of growth factors in other tissues may moderate the response compared to that observed in isolated cells. Mutation rates depend on the cell cycle times in vivo, dynamic repair processes for DNA adducts, and immune surveillance of initiated cells. Growth factors may be mitogenic in a cell suspension, but elicit homeostatic responses in the intact tissue that subsequently depress overall tissue proliferation (Jirtle et al., 1991). This paradoxical behavior, mito-stimulatory factors producing inhibition of cell proliferation in an intact organ, may occur frequently in mature tissues where little net proliferation can occur without increasing the tissue size well-beyond normal. Tissue response

modeling is in its infancy and must be more fully developed before it can be used to integrate in vitro observations for predicting full scale toxic effects in animals.

Risk Assessment: In vitro approaches then tantalize us with their promise for reducing reliance on toxicity tests in animals and improving risk assessment. However, their main use remains qualitative; they establish the potential of a chemical to elicit an effect. How will their more complete potential be realized? To my mind, the more pervasive application of in vitro toxicity results in risk assessment will only be possible as these in vitro results are combined with quantitative mathematical descriptions which integrate in vitro findings into the context of the living animal and relate the in vitro to the in vivo exposure conditions. Dosimetry models already are at a stage of development where they can be applied with in vitro results to predict behavior in animals and in people. Extrapolation based on the initial cellular interactions examined in vitro also appears possible, but more validation is necessary in these areas to provide confidence in these approaches. Predicting dose response characteristics of toxicity directly based on in vitro results is in its infancy and will only move forward if there is commitment to co-ordinated development of in vitro technologies and physiological modeling of these more complex events in vivo. Without a clear strategy describing the manner in which these in vitro studies will impact risk assessment, in vitro toxicology will continue to contribute primarily to hazard identification. This strategy is provided by the detailed biological framework provided by these physiological dosimetry and tissue response models. The partnership of quantitative modeling strategies and in vitro research promises to open an immense body of in vitro research for more direct consideration in the risk assessment process.

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DR. MELVIN E. ANDERSEN

Dr. Andersen received his undergraduate degree in Chemistry (Sc B; Brown University, 1967) and his Ph.D. in Biochemistry and Molecular Biology (Cornell University, 1971). He served in the Navy on active duty as a Toxicologist from 1971-1978 and, after leaving military service, worked at the Toxic Hazards Division, Armstrong Aerospace Research Laboratory, Wright-Patterson Air Force Base, Dayton, Ohio from 1979-1988. In his last year at Dayton, he served as Division Director. Since January 1989 Dr. Andersen has been with Chemical Industry Institute of Toxicology, Research Triangle Park, NC, serving first as Head, Risk Assessment Department and presently as Senior Scientist. His major research contributions have focused on developing physiologically-based computer models for tissue dosimetry (pharmacokinetics) and tissue responses (pharmacodynamics) of toxic chemicals of occupational and environmental interest and applying these models in quantitative human health risk assessments. He is a strong proponent of using these models in hypothesis driven research to improve experimental design and minimize the numbers of animals used in toxicological research and in developing predictive dosimetry and response models based on *in vitro* studies and limited *in vivo* experimentation. Dr. Andersen has received a variety of awards for his research, including the Frank R. Blood and Achievement Awards of the Society of Toxicology, the Herbert E. Stokinger Award from the American Conference of Governmental Industrial Hygienists, and the Kenneth B. Morgareidge Award from the International Life Sciences Institute. He is author/co-author on more than 100 research papers and 20 book chapters.

Table 1: Definitions of Some Terms

Calculated variables:	**Units**
A_t - amount in tissue	mg
C_t - concentration in tissue	mg/L
C_a - arterial blood concentration	mg/L
C_{vt} - venous blood from tissue	mg/L
Tissue Compartments:	
V_t - tissue volume	L
Q_t - tissue blood flow	L/hr
P_t - tissue partition coefficient	L blood/L tissue
Biochemical parameters:	
V_{max} - maximum velocity	mg/hr
K_m - Michaelis dissociation constant	mg/L
B_{max} - binding maximum	mg/tissue
K_b - binding dissociation constant	mg/L
k_f - first-order metabolic rate constant	/hr
k_{mm} - second-order MM binding constant	/M/hr
k_{dt} - first-order detoxification rate constant	/hr
k_{repair} - first order rate constant for adddct repair	/hr

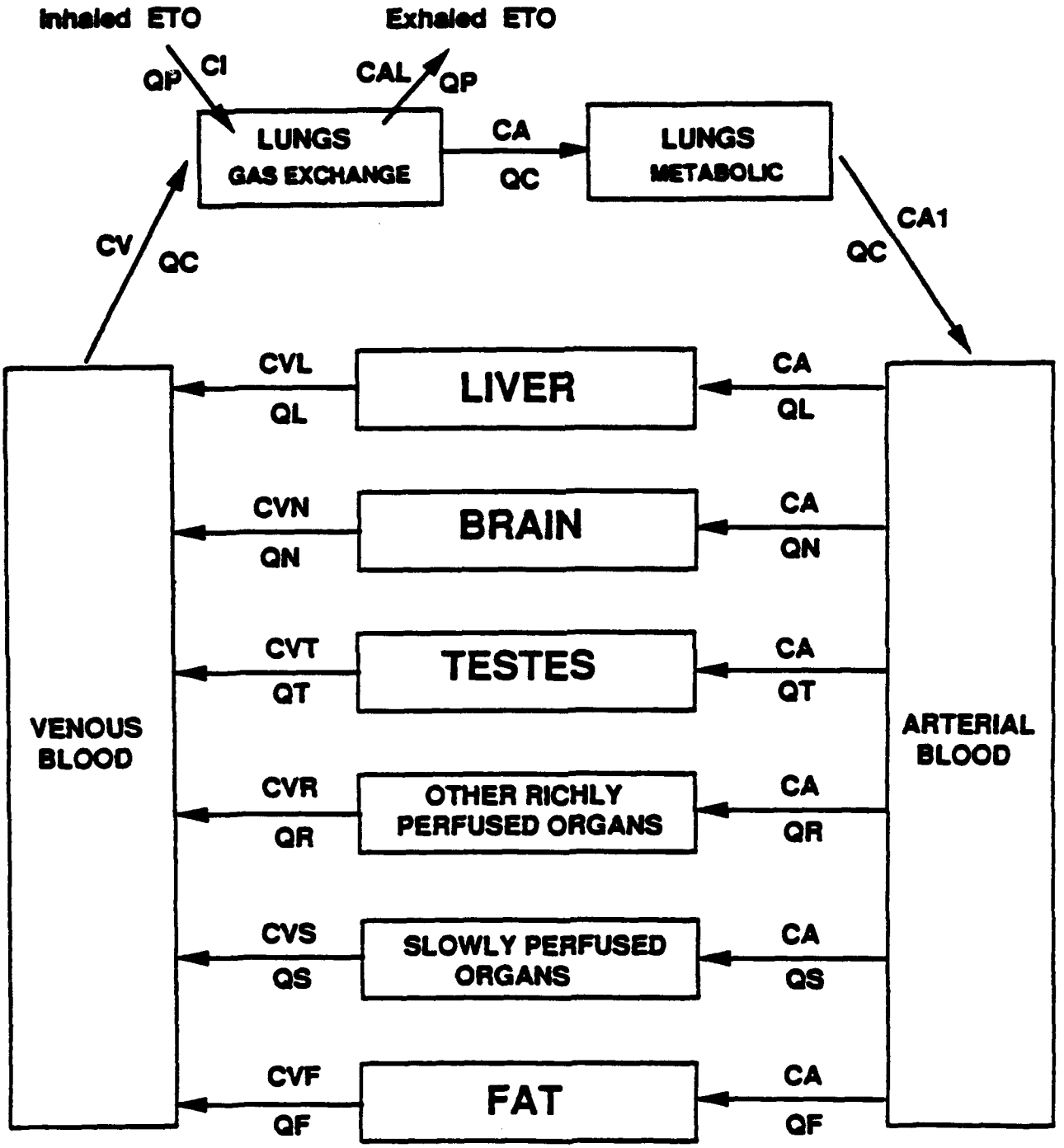
Table 2

Some In Vitro Systems Used To Estimate Parameters for Physiologically-Based Dosimetry Model

Parameters	Techniques	Tissue Preparations
Partition Coefficients minced tissue	Vial-Equilibration	tissue homogenates, cells,
	Equilibrium Dialysis Ultrafiltration	tissue homogenates, cells
Binding Constants	Scatchard Analysis Equilibrium Dialysis	purified preparations, homogenates, cells
Metabolic Constants	Spectrophotometric, Spectrofluorimetric, HPLC assays, etc.	Homogenates, subcellular fractions, intact cells, isolated perfused organs

Figure Legends:

Figure 1. A Schematic of a Physiologically-Based Pharmacokinetic (i.e., dosimetry) Model for Ethylene Oxide (EtO). Q terms refer to blood and air flow rates; C terms refer to concentrations. The concentrations each represent the concentration of chemical coming from the previous compartment; Cvi refers to the venous blood concentration exiting tissue, i. Qc,Qp,Ci, and Cal are, respectively, cardiac output, alveolar ventilation rate, inspired concentration, and alveolar concentration of EtO.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Session I: *In vitro* and Other Alternatives in Skin Toxicology

Co-Chairs: Drs. Howard I. Maibach and Francis N. Mazulli

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GENERAL OVERVIEW OF IN VITRO AND OTHER ALTERNATIVES TO SKIN TOXICITY EVALUATION

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ABSTRACT

In vitro and other animal alternative models proposed for routine skin toxicity assessment utilize various test systems, employ a variety of toxicologic endpoints, and are each at different stages of validation. During recent years, models have been developed for the assessment of: dermal irritation, dermal penetration, skin sensitization, cutaneous photosensitization, vesication, etc., with the majority emphasis being on the development and validation of in vitro alternative models to replace or reduce the number of animals required for primary dermal irritation testing. At present, no model has received regulatory agency approval as a complete animal replacement for any type of routine skin toxicity assessment. The diversity of available in vitro primary dermal irritation models, their endpoints, and their different stages of validity relative to in vitro findings provides a current example of the problems faced in the development and widespread acceptance of alternative models. Nevertheless, such models to appear to be of significant current value to dermatoxicologic research and will, no doubt, begin to play an ever-increasing role in the reduction of animal requirement for routine skin toxicity assessment in the years ahead.

INTRODUCTION

The skin, the largest organ of the body, is a complex and dynamic organ composed of an outer dermis and underlying dermis (Figure 1). Within these two primary divisions, numerous cell types and specialized adnexal structures are found. With its collection of different cellular layers, glandular components, hair follicles, vascular elements, etc., functioning interdependently, the role of the skin goes well beyond simply being a barrier to the environment. The skin also contributes significantly to the normal body function in other important ways: it has several physiological roles such as controlling water loss and regulating body heat; includes defensive roles such as protecting underlying organs, providing localized immunological defenses and photobiotic protection; has endocrine and apocrine

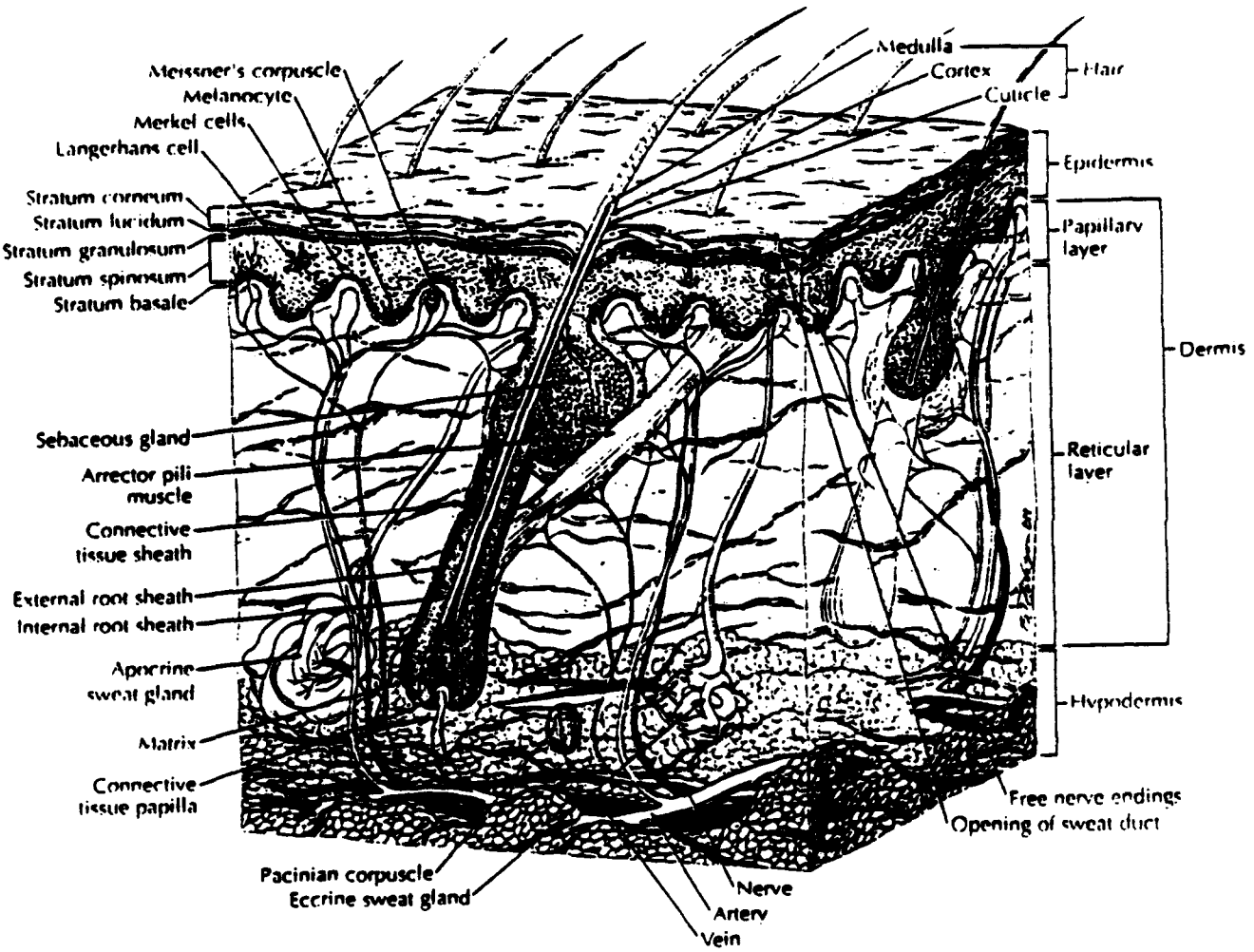

Figure 1. A composite representation of the cross sectional structure found in skin of various regions of the body. (From Chapter 1 of reference 6).

functions; and performs several important metabolic functions (i.e., keratin, melanin and collagen synthesis, carbohydrate metabolism, lipid storage and metabolism, respiration, Vitamin D synthesis and xenobiotic metabolism). The skin also contains several different types of sensory receptors which provide the nervous system with an integrated network from which to assess and respond to pain, heat, cold, pressure, irritation and other stimuli. In a 70 kg, adult human, the surface area of the skin is estimated at 1.85 square meters, of which between 0.1 and 1.0 percent is accounted for as sweat glands and hair follicles. Skin thickness is highly variable, and ranges from 400 to 600 micrometers in callous areas such as the palms of the hands and soles of the feet, to between 8 to 15 micrometers on the arms, back, legs and abdomen. Under normal conditions, the stratum corneum contains a variety of lipids and waxy esters as well as being partially hydrated. The skin generally contains about 90 g of water per gram of dry tissue, but can increase its tightly bound water content three to five times upon following direct contact with water. Because the skin is a living integument comprised of many different cell types and connected intimately with the vascular and nervous systems, the fate of percutaneously applied chemicals can be somewhat diverse (Figure 2) and the events occurring in dermal inflammatory, sensitization and corrosive effects are, likewise, now known to be fairly complex (Figure 3). Historically, animal models of various types and human testing have been used to assess the skin toxicity of various chemical substances and mixtures partially out of appreciation for the response complexity and out of convenience. In vitro procedures, on the other hand, were used to isolate and study particular aspects of the in vivo response, but have only recently started to receive serious consideration as full or partial replacements for selected in vivo models. At present, the generally accepted types of in vitro models include those that assist in:

- the selection or screening of in vivo test candidates
- the prioritization of compounds for more detailed testing or for risk assessment
- the investigation of activation or detoxification mechanisms
- the determination of dermal penetration rates or absorptive characteristics.

TYPES OF MODELS

Due to increasing interest in the reduction, replacement, and refinement of in vivo model utilization through the introduction of alternative and in vitro procedures, the number of such models is, likewise, increasing rapidly.

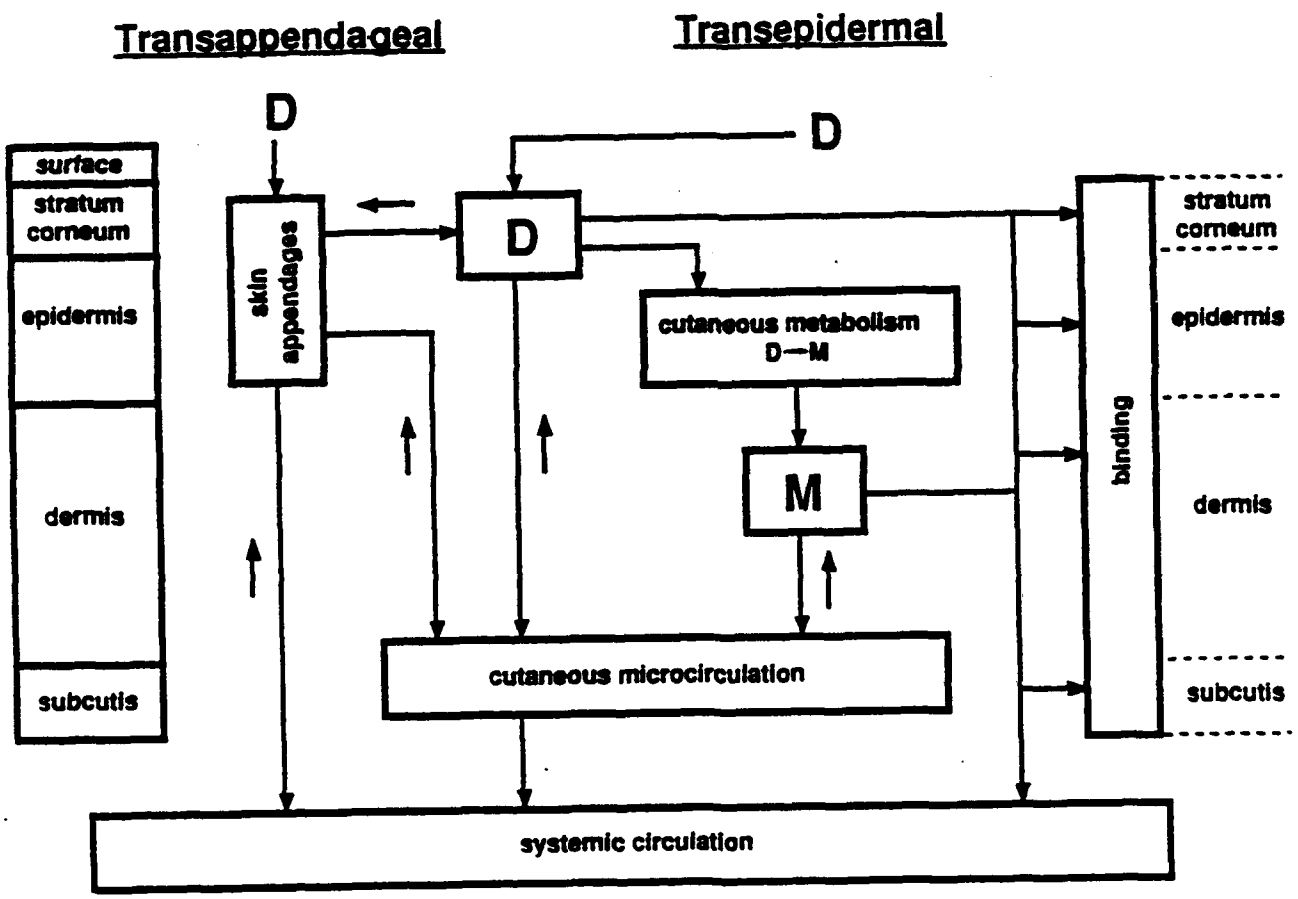

Figure 2. Schematic showing the percutaneous fate of a compound (D) following topical exposure. M denotes cutaneous metabolites of D. (From Chapter 9 of reference 6.)

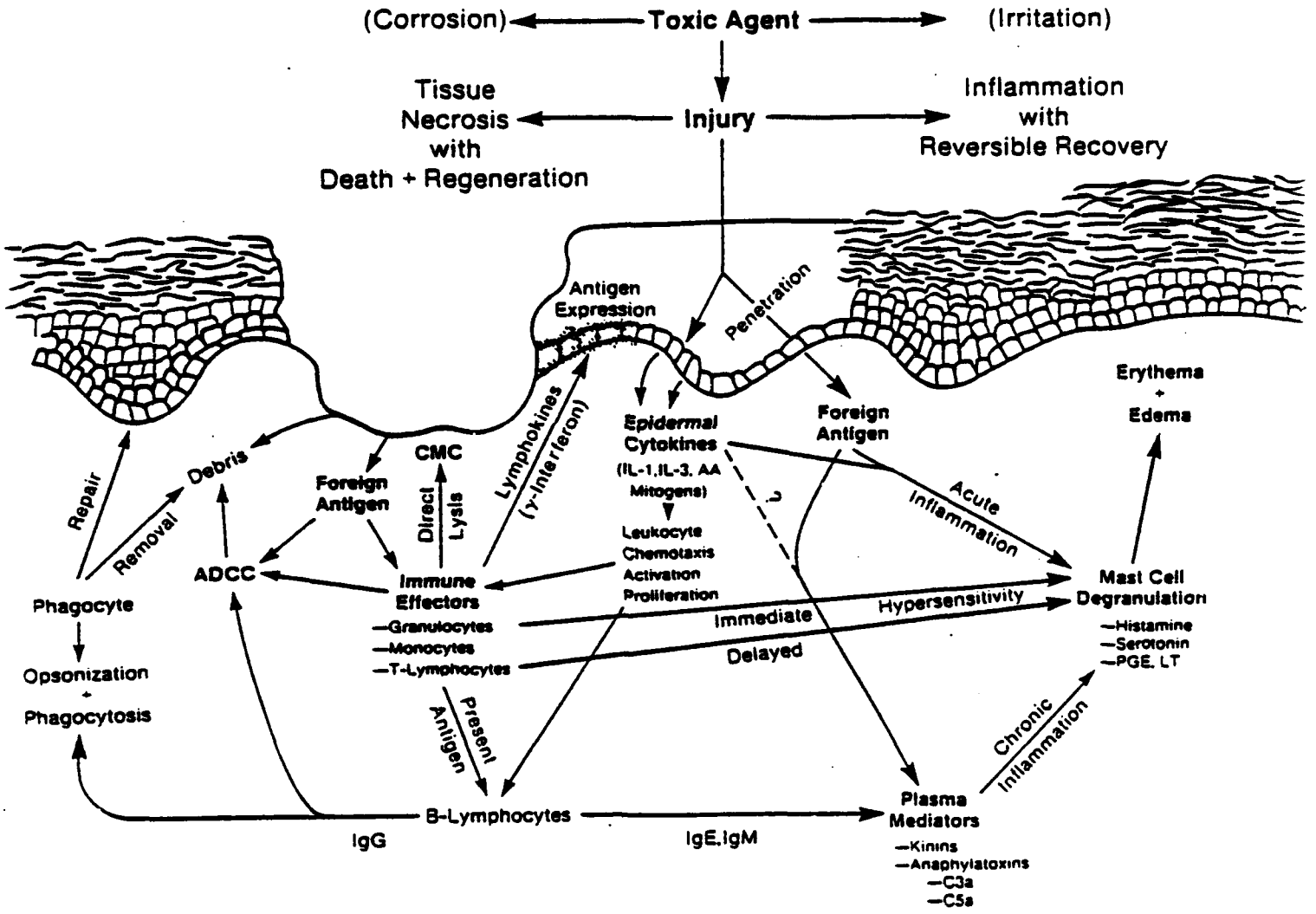

Figure 3. Illustration of the cellular an humoral mechanisms of dermal irritation and corrosion. Abbreviations: IL-1, IL-3 = interleukins 1 and 3; AA = arachidonic acid metabolites; PGE = "E" series prostaglandins; LT = leukotienes; CMC = cell-mediated cytotoxicity; ADCC = antibody-dependent cellular cytotoxicity; Ig = immunoglobins. (From S.T. Boyce, J.F. Hansbrough, and D.S. Norris, *Alternative Methods in Toxicology*, A.M. Goldberg, Ed., Mary Ann Liebert, New York, 1988.)

Fortunately, the various in vitro or alternative model types can be classified as generally being one of five different types:

1) Quantitative, Structure-Activity Relationship (QSAR) Models,

2) Biochemically based Models,

3) Cellular Models,

4) Tissue Models,

5) Lower Vertebrate Models.

QSAR Models:

These models are strictly computational in nature and require, as a minimum, the following:

- a verified database that contains toxicity as well as structural information
- a statistical technique (usually some form of correlation analysis)
- selection of key structural parameters (e.g., ring versus no ring) for the correlation
- computer technology.

In a strict sense, QSAR toxicity estimates are actually in calculo estimates rather than in vitro evaluations. The accuracy and reliability of any QSAR estimate is highly dependent on the extent and quality of the database upon which the estimate is derived. For one of the better databases which includes a total of 786 compounds, the predictive accuracy is reported to be approximately 90 percent with indeterminate estimates of about 12 percent[1]. Because QSAR databases are dependent for validation upon actual quantitative in vivo and in vitro data, it can be anticipated that the current interest in developing in vitro skin toxicity models which have as a basis some understanding of chemical structure versus biochemical target mechanics will only serve to enhance the quality of QSAR well into the future.

Biochemically Based Models:

These models are based on the concept that chemical toxicity in the skin originates from some type of deleterious interaction between a biomolecular target molecule and a toxicant. For example, it is thought that organic arsenicals exert their initiative effects as a result of inhibition of pyruvate dehydrogenase inhibition with basilar and maturing keratinocytes[2]. Surfactants, in most cases, are thought to act on membrane lipids to produce

dermal inflammation and necrosis, and sulfur mustard vesication is thought to involve stimulation of DNA repair mechanisms following DNA alkylation[3,4]. The development of successful biochemically based models usually involves the identification of molecular target(s), their subsequent isolation, and the development of a controlled test procedure using the isolated system. An example of a recently developed model which utilizes a more collective biochemical target interaction approach is the Skintex® system (Figure 4)[5]. Because this system is comprised of both a biochemically definable penetration barrier as well as a complex molecular target matrix, this system is probably best described as an advanced biochemically based test. Molecular interactions within the test system are characterized by increasing measurements of optical density. Scoring of the test results is accomplished by plotting the optical density measurements obtained with known skin irritants against their known, in vitro, primary dermal irritation index (PDII) values and then locating unknown predictions based on their respective optical density measurements (Figure 5). In its present form, the Skintex® assay has been optimized for four different test predictive requirements:

1) acidic or alkaline substances

2) high sensitivity

3) human response

4) standard chemical labelling.

Cellular Models:

These models currently utilize human keratinocytes grown in serum-free media in most instances, but models using fibroblasts, Langerhans cells, leukocytes and mast cells are also available. Cellular models, in general, provide a greater degree of molecular integration and diversity than biochemically based models. They are commonly used to assess the effects of chemicals on specific cell types, most commonly the endpoint of interest is cellular viability as determined using any of several possible assays including: neutral red dye uptake; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide or MTT dye uptake; trypan blue dye retention; propidium iodide uptake; lysosomal enzyme release; ^{3}H-thymidine incorporation; glucose utilization; etc.). There are also cellular models which involve the measurement of effects on specific intracellular targets such as DNA alkylation, membrane integrity, mitochondrial effects, etc., and models which assess effects on specific biochemical pathways, NADH depletion, ATP synthesis, glucose metabolism, protein synthesis, etc. The selection of the cell model and endpoints used to assess effects should be based on the needs of the investigation and must always take into consideration the possibility of interference by direct interaction of the test chemical with some aspect of the assay (i.e., chemical interaction with dyes used to assess viability) or indirect action with some aspect of the assay environment (i.e., pH changes in the media which affect enzymatic activity or dye color)[6]. Since human keratinocytes have recently become readily available commercially with a reasonable degree of quality

control, it is reasonable to assume that relatively standardized cellular toxicity screening models using keratinocye cultures will become popular in the near future. What remains are careful assessments of the predictive accuracy, and sensitivity and validity of these models for either generalized or specific screening use.

Tissue Culture Models :

These models may consist of either natural human or animal skin explants or cultured human or animal skin "equivalent" material. Toxicity assessments using tissue culture models generally concern themselves with effects on both specific cell types as well as on structural relationships and proliferative activity. Needless to say, natural skin is clearly superior over any of the cultured tissues in terms of its similarity of structure and functional relationship to human in vivo skin models. Natural skin models, however, due to individual and collection site diversity, suffer from a lack of providing a highly controlled and standardized testbed for routine toxicity assessment. This, combined with the rather opportunistic aspect of the availability of suitable quantities of natural human skin for test use, makes the development of routine screening tests based on human skin tissue culture rather impractical. Natural human tissue, when available, is, therefore, used as a final comparator or standard for the development of screening tests using the more controlled and supplied on demand skin equivalent technologies, which have very recently become commercially available.

In selecting a human skin equivalent material for use as the basis for a screening test, several items must be considered in addition to the obvious cost-per-test-unit issue. The desired endpoints must be carefully weighed against each model to determine model suitability for the given purpose. Skin equivalent models available commercially are likely to differ in potentially important structural aspects such as the presence or absence of a basement membrane, the number of keratinocytes in epithelial "columns", degree of cornification of the keratinocyte strata and cellular morphology of different cells used to develop the model. In a more functional sense, the models may also differ in the degree of quality control used to produce them, their structural integrity relative to natural skin, and their packaging which, in turn, may affect efficient handling of the product in screening tests.

Whether the assay uses natural human skin or a human skin equivalent, the number of possible endpoints which can be used for the toxicity assessment is much greater for tissue culture models than any other type. In addition to many of the viability, target-specific and metabolic endpoints used for toxicity assessment in the cell culture models, tissue culture models offer the ability to study both structural changes as well as cell-cell interactive effects such as inflammatory mediator release[6].

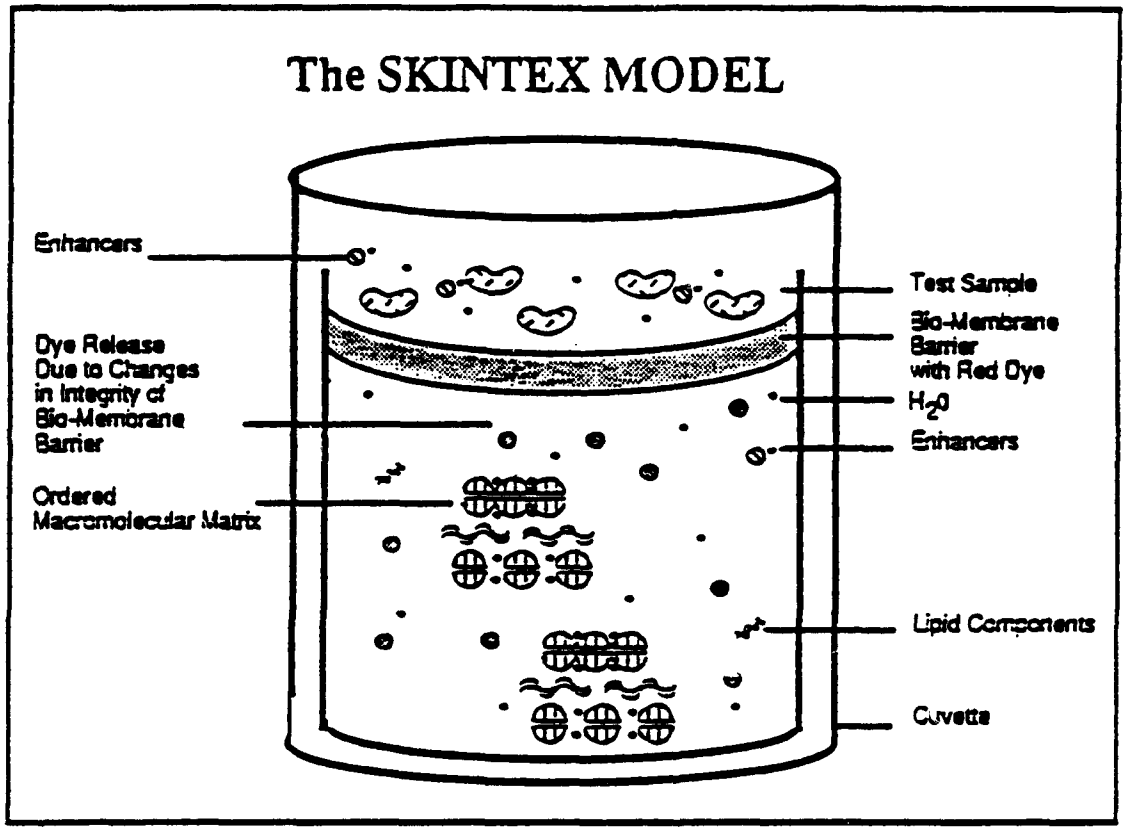

Figure 4. A schematic representation of the Skintex™ assay system. (From reference 5.)

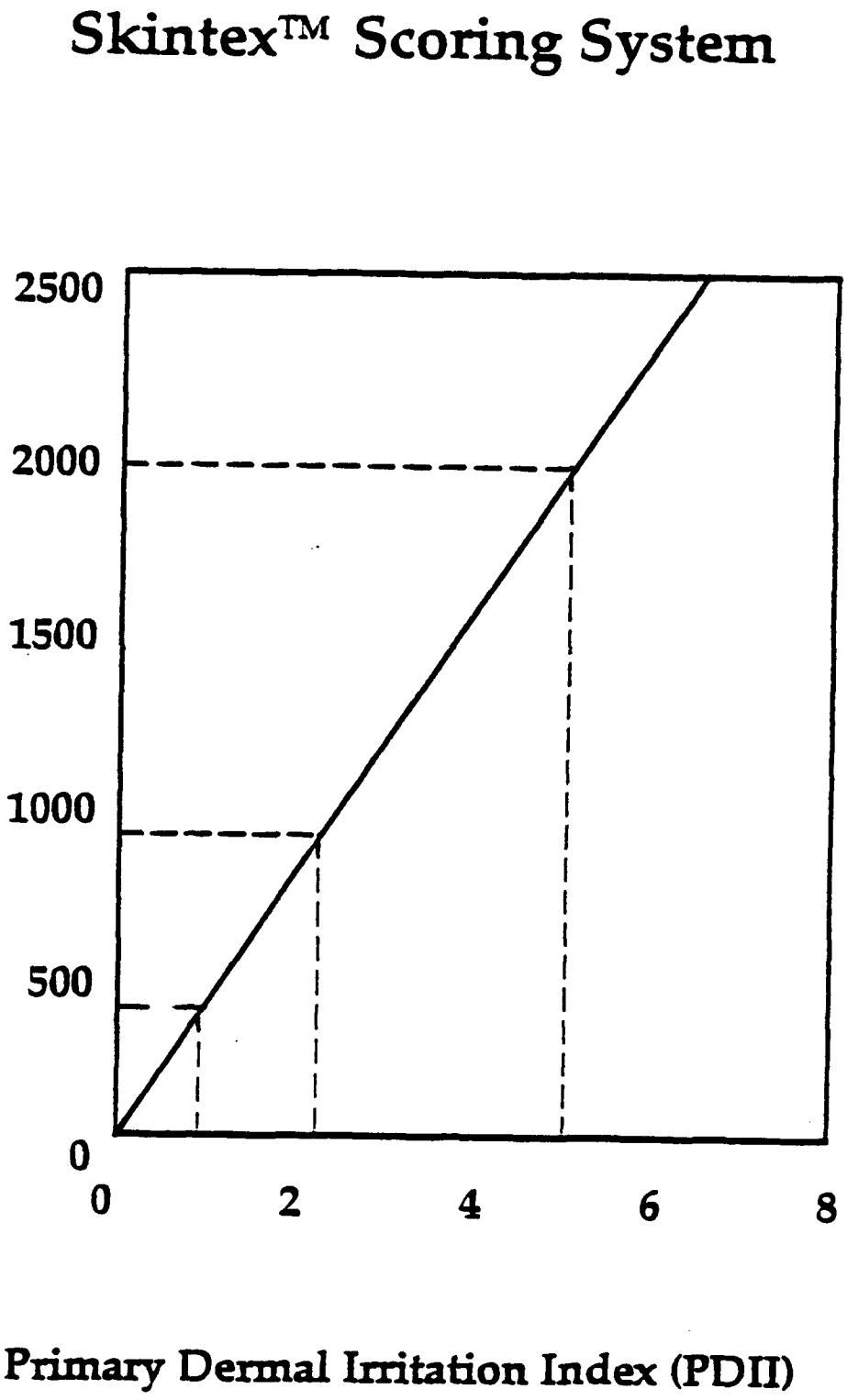

Figure 5. The Primary Dermal Irritation Index (PDII) correlated Skintex® assay scoring procedure. (From reference 5.)

Lower Vertebrate Models:

The use of a lower vertebrate model, more specifically, the chorioallantoic membrane (CAM) of a developing chicken embryo, has been proposed for use as an alternative model for the assessment of dermal and ocular acute irritation and corrosion testing. While not being an in vitro alternative model, interest in this model has been high due to its low cost and relative simplicity. Although evaluated extensively in both the U.S. and Europe as a possible dermal and ocular Draize test alternative, the model seems to be currently of greater interest in Europe as a possible alternative than in the U.S. The reason for this is not clear from a purely scientific perspective, but may be due to more success in validating the test against European in vivo test results and a significantly higher demand for the development, approval and use alternative models in Europe[7].

ASSAY DEVELOPMENT AND VALIDATION CONSIDERATIONS

Although often there are, by design, a few technical similarities between in vitro and in vivo procedures to evaluate the adverse effects of dermal irritants and inflammatory agents, many of the in vitro test systems involve, overall, several more technically complex operations relative to their in vivo counterparts. As a result, there can be many problems and sources of error which exist for such in vitro systems which are either absent from or less vexing for in vivo test systems. Because several in vitro test systems have been developed, evaluated and proposed for use as possible replacements of or adjuncts to various types of in vivo tests over the past two decades, there exists a relatively large and expanding body of knowledge concerning the nature and types of problems one can expect when developing or conducting in vitro toxicity tests. Although the technical problems associated with particular tests are routinely discussed in the literature, at scientific meetings,or between individual scientists, very few publications are available which systematically present, discuss, and summarize many of the common technical problems associated with in vitro test systems. Recently, however, a technical report was issued by the Center for Alternatives to Animal Testing (CAAT) at the Johns Hopkins School of Hygiene and Public Health as the result of findings from a technical workshop which discussed and summarized many of the technical problems encountered in the development of cell culture assays to assess chemical toxicity[8]. The report identifies several factors which may confound the results obtained from in vitro tests and notes that these factors tend to fall into two general categories: (1) those which affect biological components [i.e. cultured cells, enzymes, membranes, etc.] of the test, and (2) those which affect the dispersal and/or stability of the test material in the test medium. Category (1) includes factors which may unintentionally alter or influence the responsiveness of the biological component of the test to the inherent toxic effects of the test chemical such as:

- pH changes in the test medium resulting from addition of the test chemical,
- alterations in test media osmolarity at high concentrations of the test chemical,
- alterations in the composition of test media resulting from reactions between test chemicals and media components (e.g., protein denaturation, alteration in the availability of essential substrates or cofactors, etc.),
- confounding effects on the test system response occurring from the presence of chemicals (e.g., alcohols, dimethylsulfoxide, etc.) used as solvents for test chemicals,
- interactive effects of test chemicals and antimicrobial agents on test system response,
- complications due to evaporation of volatile test chemicals,
- interference with the endpoint measurement (e.g., the presence or formation of interfering colors in a colorimetric assay directly from test chemicals, interaction between an enzyme endpoint and the test chemical),
- influence of protein binding of test chemicals on the concentration-response curve,
- effect of test incubation time or temperature on the availability of essential nutrients,
- effect of test incubation time or temperature on the responsiveness of the test system to test chemicals,
- deterioration of test system purity and responsiveness as a result of microbial contamination, and
- physical effects of insoluble particulates, formed as a result of interactions between test chemicals and components of the assay system, on the responsiveness of the test system.

The second category, Category (2), of potential technical problems identified in the report includes potentially confounding factors related to the dissolution of the test chemical in the test media. Avoidance of problems in this category involves the examination of certain specific issues prior to actual testing to avoid the occurrence of physical/chemical problems resulting from the incompatibility of the test chemical with the biologic components of the test. The fact that many test chemicals or chemical formulations are hydrophobic in nature and that most <u>in vitro</u> systems exist within an aqueous matrix in which such test chemicals are either insoluble or nondispersive is one of the principal problems of this type which can occur. Some of the most

serious issues limiting the general usefulness of such in vitro systems stem from this fundamental problem of test chemical dispersal within the test matrix. For example, it is very difficult to establish the proper dose administered to a test system if the test chemical is either incompletely dispersed within or degraded (e.g., hydrolyzed) by the matrix. It is also difficult to determine the most appropriate means of physically introducing a test chemical into a test matrix in which is immiscible. Aggregates of non-dispersed chemicals or chemical mixtures can also have physical properties which cause mechanical damage to the test system rather than intrinsic toxicity (e.g., detergent or crystallization effects upon cellular membranes).

The incompatibility of test chemicals with components of the test media is probably one of the most significant problems encountered with in vitro dermal toxicity assays. It also appears to be one of the most common problems. This is probably because: (1) many in vitro systems require that the test chemical be introduced into some type of liquid test matrix (e.g., buffer, cell culture media, etc.) and, (2) even after pH, osmolarity and solvent effects are controlled or eliminated, degradation and/or binding of the test chemical in the test matrix can still occur.

Of course, no matter what technical problems are encountered, identified and then solved, during the development of an in vitro assay, the assay is only going to be of use if it is found to be capable of producing results with an acceptable degree of validity to some standard for which its development was intended. While it is true that significant technical problems can and do often result in problems which affect the validity of such assays, the most difficult problem currently affecting the achievement of valid results for most assays concerns the proper means by which assays are designed and determinations of validity are made.

CONCLUSIONS

The number of in vitro or alternative assays proposed for use in the evaluation of the potential for toxic chemicals or chemical mixtures to cause dermal irritation, inflammation and corrosion is rapidly growing stimulated by a continuing desire to develop valid methods for the reduction and replacement of current laboratory animal tests as well as the introduction of new bioassay procedures and technology. Proposed alternative systems which have been shown to be responsive in some fashion to known dermal irritants, inflammatory or corrosive agents can be categorized by their biological complexity. In order of complexity, these systems include QSAR procedures, biochemical assays, cellular assays, tissue culture models and lower vertebrate models. Unfortunately, none of these systems has been found to be universally acceptable for use as a routine replacement for any in vivo procedure to evaluate such dermal effects. Reasons for this are numerous and varied. To some extent, this lack of acceptability appears to involve the inherent difficulty in getting any new technology intended to replace an accepted standard generally recognized and accepted by the public, scientists,

businessmen and regulators. There is, in fact, a strong and growing interest among toxicologists to utilize alternative test systems whenever possible as replacements for or adjuncts to in vivo procedures.

Unfortunately, there are several important scientific issues related to the design, operation and validation of many of the alternatives which must be resolved before any of these systems is found to be generally acceptable as a replacement for any in vivo test procedure. These issues vary from one proposed alternative system to another. Often a test system is identified which responds as expected to some test agents and then, upon more extensive evaluation, it is found to be unexpectedly unresponsive to others.

Thus, the first step toward the development of universally acceptable assays should be to perform studies designed to identify any significant variables which must be controlled in order to establish an acceptable level of response and quality control.

Despite the growing number of proposed tests, the acceptance of such tests by toxicologists as valid replacements for animal tests has been cautious. This situation is in large part due to the need to conduct more comprehensive and impartial validation studies involving a wide spectrum of chemicals with known in vivo dermal toxicity and for the establishment of formalized criteria to define the circumstances under which a particular alternative test is considered valid. As such, there exists a need for the development of a generally acceptable validation protocol for the development and acceptance of such procedures, if they are to become widely accepted. Unfortunately, in addition to the inherent problems with the tests themselves, the funding and organization of large-scale validation studies is currently not widely available for many of the potential users of such tests, and progress toward acceptance has been slow. It is anticipated, however, that due to the increasing desire among scientists, politicians, businessmen and consumers to identify suitable alternative tests to assess dermal toxicity, validated alternative procedures will emerge within a few years for the assessment of specific types of cutaneous toxicity endpoints which will be widely accepted by toxicologists.

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IN VITRO SKIN ABSORPTION/METABOLISM TECHNIQUES FOR DERMAL TOXICOLOGY

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A knowledge of skin absorption and metabolism is required for an accurate risk assessment following skin exposure to a toxicant. Use of *in vitro* methodology permits collection of human data for chemicals that are too toxic for clinical study. Studies conducted with animal skin will generally give an overestimate of human absorption. The metabolism of human skin may also differ from that of animal models. Recent advances allow simultaneous measurement of skin absorption and metabolism in skin kept viable in flow-through diffusion cells (Collier et al., Toxicol. Appl. Pharmacol., 99, 522-523, 1989; Bronaugh et al., Toxicol. Appl. Pharmacol., 99, 534-543, 1989).

Predictions of percutaneous absorption can be made from physicochemical data (octanol/water partition coefficient and molecular weight). The accuracy of absorption estimates may be improved by grouping chemicals into structurally related classes (for example, aliphatic, monocyclic, polycyclic).

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IN VITRO DERMAL TOXICITY ASSAYS: VALIDATION WITH HUMAN DATA

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Abstract: The following describes currently available in vitro assays designed to predict irritant/toxic capability of compounds applied to the skin. Aspects of the validation process of these assays are discussed, particularly identified limitations. Validation projects using in vivo human data are discussed and examples given.

In the past two decades, the scientific community has witnessed greater interest in the development of alternative in vitro assays for predicting ocular and dermal toxicity. The reasons for this relate mainly to criticisms of the most widely utilized test for ocular/dermal toxicity--the rabbit Draize test.[1] The criticisms directed against the in vivo rabbit test include (1) the subjective nature of the visual scoring system employed, (2) the questionable ability to extrapolate results to man,[2,3,4] (3) the fact that the test is time consuming and costly, and (4) the fact that the test results in animal distress.

There currently exist a number of in-vitro alternatives designed to predict the dermal toxic/irritant capability of compounds in man. These alternatives are based on a variety of methods including cell culture, physico-chemical analysis, microorganism studies, isolated tissue techniques, and computer modeling. Because the process of dermal irritation is complex and its various mechanisms have yet to be fully discerned, no single parameter has emerged as a "best predictor" of irritation. The myriad of in vitro assays, therefore, utilize a wide array of endpoints designed to be predictive of irritation/toxicity. Such endpoints include cell death, decreased cellular metabolic function, decreased cellular protein production, decreased mitochondrial function, decreased fluorescence capability, decreased motility of simple protozoa, histologic changes, release of intracellular enzymes, release of inflammatory mediators, damage to vascular systems and protein denaturation. The utility of these endpoints are made more clear in the following paragraphs which discuss individual test methods.

Cytotoxic tests which use cell culture systems include the neutral red dye uptake assay, the MTT dye uptake and reduction assay, and assays which measure the release of cellular proteins or inflammatory mediators. The neutral red dye uptake assay uses a dye which is taken up by viable cells, and retained in lysosomes. Perturbations by cytotoxic agents that result in damage to either plasma membrane or lysosomal membrane uptake systems therefore result in decreased dye uptake. The endpoint for this assay is known as the NR_{50} and represents that concentration of toxicant which reduces by 50% the uptake of dye as compared to control cells.[5] The MTT assay correlates cell viability to both intact mitochondrial function and intact plasma membrane uptake processes.[6,7] This system uses a tetrazolium salt which in viable cells is reduced to a blue formazan product by the action of mitochondrial enzyme systems. Cytotoxic agents which interfere with

mitochondrial function cause decreased formation of the blue formazan product. The measured endpoint for this system is an MTT_{50} which, like the NR_{50}, represents that concentration of toxic agent which reduces the formation of formazan product by 50%. Other cell culture based systems utilize the release of cellular proteins, such as lactate dehydrogenase, beta glucuronidase, or alkaline phophatase.[8,9] The release of these intracellular enzymes is representative of damage to plasma membrane retention systems by cytotoxic agents. Measurements of inflammatory mediator release, such as arachadonic acid, prostaglandins, and leukotrienes represent the cells' response to perturbation by irritating (but not necessarily lethal) substances.[10,11] These mediators are synthesized by viable cells and released to the extracellular matrix as part of the inflammatory response to irritating substances. Finally, other cell culture based systems have correlated cell viability to such endpoints as total cellular protein content (Kenacid blue dye method),[12] glucose utilization (i.e. depletion of glucose from the extracellular medium),[13] and changes in cellular metabolic activity. The latter system is marketed as the silicon microphysiometer, and represents a sensitive method capable of detecting small changes in the metabolic activity of a monolayer cell system.[14] The principles of this device are based on the premise that cellular metabolic activity is reflected by the concentration of extracellular, acidic byproducts such as lactic acid and carbon dioxide. Changes in the metabolic activity of the monolayer (as a result of toxic/irritant effects) are therefore represented by changes in the pH of the extracellular medium. These subtle pH changes are measured by means of a silicon-based electrode known as a light addressable potentiometric sensor (L.A.P.S)

All of the aforementioned cell culture-based methods generally use monolayer cell systems; however, there now exist commercially available multilayer cell systems, collectively known as skin equivalents.[15,16] Such systems consist of multilayers of dermal fibroblasts or keratinocytes grown on a variety of substrata. In many of these commercially available models, the cells are keratinocytes or fibroblasts derived from human tissue (i.e. as a result of circumcision or breast reduction surgeries). Some of the systems contain a stratified, differentiated epithelium, which arises as a result of raising the system to the air-liquid interface. In general, the same endpoints utilized for the monolayer systems can also be used in these multilayer skin equivalents. In the future, the incorporation of other cell types, such as mast cells, macrophages, neutrophils, etc. will be the goal in making these systems even more predictive of the in-vivo situation.

Other in vitro assay systems utilize the chemical processes of bacteria as a measure of toxic effect. The Microtox® system uses the luminescent bacterium *Phosphobacterium phosphoreum*. Toxic agents generally cause a decrease in the fluorescence capabilities of this bacterium, the degree of which can be quantitated and correlated to degree of toxicity.[17] A similarly based system uses the ciliated protozoan *tetrahymena thermophila* and correlates toxic effect to the endpoint of decreased motility.[18,19]

Since the primary events in inflammation (i.e. erythema, heat, edema) are dependent upon vascular processes, it follows that investigation of the effect of irritant/toxic compounds on a vascular network would comprise a suitable in-vitro alternative. Such a model is represented by the choriallantoic membrane system (CAM).[20,21] This system uses fertilized chicken eggs, whose vascular network (or CAM) is exposed by cutting a small "window" into the shell. Test compounds can then be applied directly to the CAM and their effects graded by noting visual changes in the blood vessel network (i.e. hemorrhage, injection,coagulation).

Physico-chemical models for the prediction of dermal toxicity are rare. The reason for this relates to the paucity of knowledge regarding the effects of toxicants at the molecular level. One model, the Skintex™ dermal assay system, uses protein denaturation as an endpoint.[22] The ability of toxicants/irritants to disturb the ordered array of protein components in the system is measured spectrophotometrically. This model provides a quantitative response to materials that may produce irritation by protein binding, enzyme

inactivation, and a variety of other pathways where macromolecular confirmation is altered in the initiation of dermal irritation.

All of the above assays are currently in a stage of validation, the goal of which is to assess both their reproducibility (i.e. degree of interlaboratory variation) and their relevance (i.e. to what degree are these tests predictive of in vivo irritation/toxicity?).[23] The assessment of relevance, therefore, is most often made by comparing the in vitro result to some in vivo result. Currently, the majority of validation projects use the Draize rabbit assay as the in vivo arm of the correlate; however, the question arises as to should the "standard" for in vivo irritancy reside in a species other than man? The reasons for the use of rabbits in toxicity testing are obvious; however, their use has been criticized due to questionable ability to extrapolate results to man and other species.[2,3,4] With this in mind, it makes sense that at least some aspect of the validation process should attempt to correlate the results of in vitro dermal toxicity tests to in vivo human data.

Such has been the aim of our laboratory. One example of this approach compared the irritant capabilities of the irritants benzalkonium chloride, trichloroacetic acid, phenol, and hydrochloric acid in the human and in the Skintex™ dermal assay system. The Skintex™ system was fairly sensitive in its ability to predict the irritant potential of these compounds in man. (sensitivity 82%, specificity 71%, positive predictive value 82%).[24] Additionally, the in-vivo dose response curves for each of the 4 substances was compared to the in-vitro dose response curves, and correlation coefficients calculated. The in-vitro dose response for benzalkonium chloride (R^2=0.987) and phenol (R^2=0.994) were strikingly similar to those generated in-vivo, possibly indicating that the mechanisms of action in-vivo and in-vitro are similar for these two compounds.

Such a comparative approach using in vivo human data will no doubt enhance the ability to assess the true relevance of a particular in vitro system; however, this approach is not without its limitations. Probably the most significant weakness of this approach is the fact that one is limited to using fairly innocuous compounds in the in vivo arm of the comparison. We may therefore be skewing the relevance assessment to less irritating/toxic compounds. On the other hand, it is generally thought that if a test is sensitive enough to detect differences in the toxic potential of compounds which are in a narrow range of irritancy, it is most likely capable of making more broad-ranged assessments. The veracity of the latter statement will no doubt be borne out by future validation projects.

Other problems encountered in the process of validation which are more general and are irrespective of the in vivo species used include the following (1) How can we make an assessment of a particular in vitro assay's ability to predict both acute and chronic irritation? (2) Similarly, what in vivo exposure period should be used to compare results.? 24 hours? 72 hours? (3) Should the in vivo exposure period be an open or closed test? and (4) How do we make the in vivo data more objective?

We believe that one solution to the above questions would involve a standardized approach to making in vitro/in vivo correlations. Such an approach would test a reference set of compounds both in vitro and in vivo. The in vivo test, whether performed on animals or humans, would involve assessments of both acute irritancy (i.e. after 24 hours) and cumulative irritancy (over the course of 21 days). Also, the in vivo irritancy test should be performed under both open and closed conditions. Finally, in addition to visual assessments of irritancy (which tend to be subjective), more objective measures of irritancy (such as transepidermal water loss, blood flow, chromatography, and conductance) should also be made. By proceeding in such a manner the in vitro result can be compared to an in vivo data base which encompasses a number of variables (time exposure; mode of application to the skin; different measures of erythema; different measures of barrier disruption). In vitro/in vivo comparisons are therefore more sound, and an improved assessment of the in vitro assay's relevance is achieved.

What then is the future for in vitro assays? First, it is apparent from already completed validation steps that a single in vitro assay will not be capable of predicting the dermal

irritancy for all classes of compounds. It seems unlikely that one assay could be predictive for the array of vastly different chemical structures which induce irritation by as yet undefined mechanisms. Rather, batteries will be employed which use single in vitro tests, each test differing as to the molecular/physiological endpoint measured. Secondly, as confidence in the reproducibility and relevance of these in vitro alternatives increases, we will see a reduction in the number of animals utilized for toxicity study. The total elimination of animal use for toxicity study is most likely far in the future. Finally, as our experience with the array of in vitro assays increases--each test with its distinct physiological/molecular endpoint--our knowledge concerning mechanisms of dermal irritation and toxicity will expand concurrently.

CONCLUSIONS

In order to protect humans from substances, skin irritation tests must be performed. At a minimum, the new tests must be sensitive enough to characterize the potential degree of irritation. In future, the developing skin systems may also provide information about the mechanism by which a substance causes irritation.

It is unlikely that an *in vitro* system could ever be developed to mimic the complex cascade of reactions that occur in the human skin. However, an *in vitro* system utilized as an initial screening device would permit the use of a minimum number of animals for skin tests and simplify the process by which new compounds are developed.

Appropriate use of human irritancy data with standard compounds, utilizing occlusive and non-occlusive dosing, should permit a facile and realistic correlation between the in vitro assays and likely human experience. As of the present, *in vitro* irritation systems are entering a validation phase. Criteria must be established permitting identification of standards, i.e., how irritating are model compounds in man (or animal)? Our personal impression suggests that the data must be clearly related to method of application (occlusive or open), single dose (primary irritant) or multiple dose (cumulative irritation); and anatomic site (face responding differently than back).

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Dr. Maibach is Professor and Vice Chairman of Dermatology at the University of California, School of Medicine, San Francisco, California (UCSF). He joined the faculty of UCSF in 1962 after receiving his M.D. from Tulane University, New Orleans in 1955, and internships at William Beaumont Army Hospital, El Paso, Texas, University of Pennsylvania Hospital, and Walter Reed Army Hospital (in neuropsychiatry).

His bibliography includes more than 1000 publications and 40 books. His most active fields of research dermatopharmacology, dermatotoxicology and exogenous dermatitis. He has been doing human subject research for 25 years. He has been on numerous committees and is a consultant to government, academia and industry worldwide.

SKIN PENETRATION (Historical)

Francis N. Marzulli

Systematic studies in skin penetration by topically applied chemicals were undertaken by British investigators in the 1950's. Treherne, followed by Ainsworth and Tregear conducted diffusion studies on isolated pieces of skin, using it as a complex membrane. They focused on the permeability constant as a measure of the capacity of a chemical to penetrate skin.

Originally, we thought of the skin as an inert protein-lipid structure whose effectiveness was largely due to the outer keratinous barrier structure. More recently, attention has been directed to the metabolic potential of the underlying living epidermis.

In vivo studies at first employed pharmacologic endpoints as a measure of skin penetration. One technique that is still used involves the blanching effect of topically applied steroids as an indirect semi-quantitative measure of skin penetration. This method was championed by Stoughton.

Over the years, work with both isolated skin and with skin in vivo has provided a better, but still incomplete picture of events involved in the skin penetration processes.

The arrival on the scene of relatively inexpensive radiolabeled chemicals enabled a fuller development of both in vivo and in vitro techniques for studying skin penetration.

Maibach, Feldman and coworkers conducted skin penetration studies on human subjects in vivo. They reported their results in terms of the percent of applied chemical that penetrated the skin.

Blank, followed by Scheuplein expanded our knowledge of skin penetration using in vitro techniques with skin as a membrane. Blank did his early work on contract with Edgewood and Scheuplein did his at Edgewood as a Chemical Corps officer at the Medical Laboratories.

More recently, Bronaugh (of FDA) has focussed attention on metabolism as an important component of the skin penetration process. Rivier uses the porcine skin flap, and, Anderson, Clewell and Mc Dougal (of Wright-Patterson AFB) favor pharmacokinetic modeling as the approach for the future.

A comprehensive review of this subject has been developed in a document recently released by the EPA. It contains up-to-date skin penetration information needed to develop a risk assessment document.

There are of course many others besides those mentioned, who contributed to our present understanding of percutaneous absorption.

This brief introductory history is intended only to whet the appetite of investigators who seek alternatives to present methodologies.

We now know something of the complexity of the skin barriers and properties of the stratum corneum that relate to penetration by fat-soluble and water-soluble compounds.

We have some knowledge of the effects of occlusion, hydration, temperature, molecular size, age, and skin site.

Studies in animals and in humans, in vitro and in vivo have all contributed to our present understanding of the processes that involved in skin penetration.

FRANCIS N. MARZULLI, M.A., Ph.D.

Dr. Francis N. Marzulli attended St. Peter's College, Jersey City, graduating in 1937 with a B.S. degree in biology. He received his masters and doctoral degrees in 1940 and 1941 respectively, from Johns Hopkins University, Baltimore. Dr. Marzulli has worked as a biologist for the U.S. Fish and Wildlife Service (1941-43); as chief toxicologist (1943-46) and senior scientist, of the Dermal and Ocular Toxicology Branch (1963-80) with the FDA; and as senior program officer of the National Research Council (1980-88) at the NAS. For the last three years, Dr. Marzulli has served as science advisor to the EPA in dermal toxicity. He has also been advisor to the Society for Comparative Ophthalmology, and an OECD representative for the Canadian FDA. Throughout his career, Dr. Marzulli has taken several teaching and lecturing assignments, has written nearly 100 scientific papers, and is co-editor of the test, "Dermatotoxicology." Dr. Marzulli is a member of the Society of Investigative Dermatology, the Association for Research in Vision & Ophthalmology, the Society of Toxicology, the American College of Toxicology, the Society of Cosmetic Chemists, the Society of Sigma Xi, and the Society of Dermatologica Polona (honorary).

ALLERGIC CONTACT DERMATITIS (Historical)

Francis N. Marzulli

Allergic contact dermatitis (ACD) is, as the name implies, a dermatitis or inflammatory skin response produced by skin contact with an allergenic substance.

Allergic contact dermatitis, as distinguished from irritant contact dermatitis, is a delayed, immune response that results in erythema, edema and vesiculation. The skin response resulting from contact with poison ivy is a good example of this effect.

The first requirement for the production of ACD is that the allergenic substance penetrate the horny layer of skin and enter the viable epidermis where it makes contact with and binds to Langerhans' cells. These are dendritic cells in the epidermis. The Langerhans' cells direct the allergen to a regional lymph node where interaction with T lymphocytes initiates a process that results in abundant replication of sensitized T lymphocytes. This constitutes the induction phase of the sensitization process. The next contact with the allergen results in elicitation of a hypersensitive skin response. That is, the skin responds to a lower concentration of allergen than was required prior to induction.

The fact that the immune system is involved and a two step process, induction and elicitation, is required in ACD, makes it more difficult to simulate this process in an alternative system that lacks the complexity of an immune system.

The patch test in some form provides the main approach to assessing ACD. Jadassohn in 1895 is thought to be the first to formalize the patch test as a means of producing skin disease on a small scale, by applying a test chemical under an occlusive patch to identify those chemicals that are allergenic. A patient of Jadassohn with hypersensitivity to mercury was demonstrated to be allergic by means of a patch test. In this case the patch test was diagnostic for a subject who was already sensitized.

The patch test can also be used in a two step procedure to evaluate whether a chemical is allergenic. In this type patch testing, induction of ACD is accomplished by applying (repeatedly) a high concentration of suspected allergen. After an appropriate time frame, during which immunologic events take place, the elicitation or challenge phase is carried out with a non-irritant concentration to find out if the test material is indeed allergenic.

Other early workers in this field were Landsteiner and Chase who suggested a role for sensitized lymphocytes in ACD. Their work was followed by the development of provocative tests in both animals and humans to identify substances with a proclivity to sensitize. This work led to information on sensitization frequency and the concentrations required for these ACD effects.

It was early recognized that induction of ACD could be accomplished by employing frequent contacts with the test allergen. Schwartz and Peck, and, Shelanski developed tests based on this type of repeated insult. It was also recognized that high concentrations of a suspect allergen at induction could also be effective. This is the approach that Draize used. Kligman used sodium lauryl sulfate (SLS) to assist in the induction process. When sodium lauryl sulfate is applied to skin it provides subtle damage that enhances skin penetration of topically applied substances. By this means, a larger amount of the penetrant reaches the immune system thereby facilitating the induction process. The SLS may act like a higher concentration of the test material.

Guinea pigs became the first animals of choice for test purposes. Draize suggested delivery of the test allergen by intradermal injection. Buehler introduced essentially a modified human patch test method using guinea pigs as surrogates. Magnusson and Kligman employed Freund's complete adjuvant to enhance the responsiveness of the animals. More recently, mouse ear swelling tests have come into vogue. The lower cost of purchasing and maintaining mice and the greater use of mice in other immunological work has probably hastened this development.

TOWARD A PREDICTIVE MODEL FOR ALLERGIC CONTACT DERMATITIS

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Recent studies of transdermal transport have identified partitioning (LogP), polarizability (MR) and H-bonding (HBA, HBD) as key mechanistic factors. Allergic contact dermatitis (ACD) is generally ascribed to direct or metabolically induced reactivity in the viable epidermis leading to an immune response against the modified protein. By analogy with drug action, it seemed to us that supporting factors involving site delivery should also be important. As a preliminary study to explore this hypothesis, we selected a group of strong ACD irritants and a group of cosmetically innocuous chemicals having similar functional groups. These were separated successfully by linear discriminant analysis and by two-valued multiple regression. The two methods are complimentary and support identical factors. In brief, the expected transport and binding factors combine significantly with 2-valued substructure descriptors related to reactivity.

Introduction

One of the most complex interactions of living skin with chemicals is expressed in the phenomenon of allergic contact dermatitis (ACD). A protein reactive chemical (hapten) or a metabolic precursor (prohapten) first modifies a structural protein in the viable epidermis. In those cases where an ACD response occurs, this initial challenge activates the immune system in the local lymph nodes. These nodes are responsible for draining edemous fluids when local tissues are inflamed. During an induction period, antigen recognition of the modified protein and complimentary templating of the T-lymphocyte receptor site takes place. Following sensitization, the T-lymphocytes proliferate in the lymph nodes and await a second challenge of the same chemical. Unlike the first challenge, which found the immune system

unprepared, the second contact can lead to a severe inflammatory reaction accompanied by erythema and edema. Under controlled conditions, the ACD response can be reliably demonstrated in the guinea-pig maximization test[1], the occluded patch test (Buehler)[2], the guinea-pig optimization test[3,4] and more recently, the local lymph node assay of Kimber et al.[5] There is reasonable but not perfect concordance among these tests for moderate to strong ACD response.

The general complexity of the process involving in vivo metabolism and the immune system response is well discussed by Dupuis and Benezra[6]. On first inspection, the ACD problem would appear too complex and too broad in chemical scope to be approachable by SAR analysis. However, recent studies in our laboratories have shown the importance of transport and binding factors in the percutaneous absorption of drugs and ordinary chemicals[7]. If such factors are important in the delivery of ACD active compounds to the response sites in the epidermis, then SAR analysis in some form is appropriate. It was clear from the outset that regression analysis of graded ACD responses was not likely to succeed. We felt, however, that if diverse compounds having known strong or weak response could be selected that a discriminatory model based on mechanistic factors could be developed. This paper represents our initial efforts to develope such a model.

Candidates, Data and Descriptors

Candidates expressing moderate to strong ACD responses were selected from the INPRET database of Volker Ziegler[8]. The database contains approximately 900 entries. However, many are multiple entries (formaldehyde, 20 entries) and many are trade mark chemicals of uncertain indentity. When sorted on strong allergens (rating=3), there are only 74 chemicals. Elimination of inorganics, salts, dyes, corrosive and very reactive chemicals reduces the set to 18. We selected 12 of these for chemical diversity and by a similar process, 12 more that were moderate allergens (rating=2). We now have 24 diverse chemicals that are clearly classified as moderate to strong ACD allergens.

In order to provide an equivalent class of non-allergenic compounds, we explored entries in the Givaudan Aroma Catalog and the CTFA Cosmetic Ingredient Dictionary. The protocol for selection of 24 non-allergenic compounds was similarity in molecular structure and the avoidance of perfect substructure descriptors. A perfect descriptor is one that appears exclusively in one class and therefore guarantees separation on a trivial basis. How well we succeeded can be judged by the substructure count for the ACD/non-ACD classes: H-bond acceptors, 70/57; H-bond donors, 28/34; aryl, hetero-rings, 27/21; aldehydes and esters, 10/11; unsaturation, 11/8; alkanols, 11/6; phenols and anilines, 15/3. It can be seen that we succeeded in most categories with the exception of the prohapten phenol and aniline structures.

Descriptors for these 48 chemicals fall into three categories: continuous, categorical and ordinal. Continuous descriptors are used to describe mechanistic behavior based on subtle differences between molecular structures. Under this category are the transport and binding descriptors that dictate the behavior of molecules as they pass through and partition among different bio-organic phases. Primary among these are LogP(octanol/water) which frequently correlates with transport and non-specific binding[9,10] and molar

refraction (MR) which correlates the polarizability (London forces) of a molecule sliding over a molecular surface (e.g. protein)[11]. As LogP(o/w) is calculable[12], it is easy to calculate the partial LogP's of the lipophilic (PL) and hydrophilic (PH) substructures. In recent binding studies, these descriptors have proven valuable when the binding polymer interacts preferentially with one class of the substructures. Categorical descriptors are set to (1) for the presence of a substructure and to (0) for its absence. They are also known as dichotomous descriptors or indicator variables in the sense that statistical significance indicates the presence of the effect being tested. Substructures selected for this study are those expected to react directly with skin structures (haptens) and those with potential for metabolic activation in the epidermis (prohaptens). Examples of the hapten structures are ICHO, ICONJ and IX representing aldehydes, conjugated double bonds and reactive halides. Prohapten structures are represented by IOH, IAROH and IARNH2 for aldehyde precursors (primary alcohols) and quinones (pheols and anilines). It must be remembered that these are test descriptors with no a priori significance. The last category (ordinal) is mechanistic in nature as it tests for the possible significance of hydrogen-bonding of these 48 structures to protein domains in the stratum corneum and epidermis. Both descriptors are based on ordinal counts of the free electron pairs on O and N (HBA, acceptor) and the number of N-H and/or O-H bonds (HBD, donor). In sum total, these descriptors cover all of the anticipated effects of binding, delivery and ACD reactivity. The categorical reactivity descriptors are crude at best, but we are not looking for details at the quantum chemical level. We will be thoroughly satisfied if these descriptors implicate the mechanistic factors and provide a reasonable model for the a priori estimation of ACD potency.

Statistical Methodology

Considerable experimentation with statistical methods led us to select two-class multiple regression analysis as the method of choice. The results obtained are completely consistent with those from linear discriminant analysis and there is no need for a redundant discussion of this approach. Two-class regression is a seldom used procedure in QSAR[14] and was unfamiliar to these investigators at the outset of this problem. The statistical meaning of r^2 (explained variance), s (equation standard deviation) and F (Fisher distribution)[measures equation strength] are nicely discussed by Lachenbruch[15]. The procedure is at its best when the two classes are equally populated or nearly so. Unlike discriminant analysis, it cannot be extended to three classes. Therefore, we cannot expect the procedure to separate strong, moderate and non-allergenic; strong and moderate must be considered together as a single class. The advantages we found in using the regression approach were the statistical strength measures of the descriptors (T-values), the overall significance of the equation (r^2, s, F) and the ability to plot the residuals to determine exactly where each compound fell.

Results and Discussion

By a process of backward selection, the least significant variables were deleted, resulting in a highly significant regression model.

ACD (n = 24) Class = 1

NON (n = 24) Class = 0

	Coefficient		T Value
CLASS =	0.048	MR	3.58
	-0.698	PL	5.35
	-0.390	HBD	3.00
	0.983	IHET	3.61
	1.168	ICONJ	5.87
	0.257	IX	2.26
	1.367	IAROH	5.02
	1.228	IARNH2	4.41
	0.928	IOH	4.58
	-0.635	(intercept)	

n = 45 s = 0.296 r^2 = 0.727 (0.853) F = 10.34

The dichotomous descriptors include both hapten and prohapten substructures in addition to IHET for heterocycles not known to be either. All have positive coefficients, meaning that these structures contribute to the ACD classification (Class=1). The weakest of these is IX where halogen reactivity extends over an enormous kinetic scale. This may be a poor discriminator as compounds such as beta-bromostyrene are cosmetically inert. Of the two ordinal descriptors, only donor H-bonds (HBD) proved significant. The negative coefficient indicates that H-bonding in the stratum corneum retards the delivery of these compounds to the viable epidermis. In the continuous descriptors, PL correlated much better than LogP(o/w) and its strong negative coefficient clearly suggests that lipophilic groups reduce reactivity with proteins and metabolic enzymes by weakening binding and slowing partitioning into protein domains. The positive correlation with polarizable size (MR) is most interesting as the strength of an immune response will clearly depend on the bulk of the abnormal structure targeted for antigen recognition and templating. A case in point is formaldehyde which reacts avidly with skin proteins and yet, is borderline in ACD response. A simple scrutiny of the two classes shows that the active set tends to be larger (MR=30.0-93.0) than the inactive set (MR=23.4-58.0).

Three outliers were removed from the final equation (n=45). Geraniol is the trans-isomer of nerol, a confirmed cosmetic ingredient and has exactly the same descriptors in this study. Both are predicted to be non-allergenic, as expected. If geraniol is truly a moderate ACD chemical as indicated in the INPRET database, then the difference must be conformational and lies outside the scope of this study. Glycidic benzoate, a moderate ACD chemical, is underpredicted by this equation. It is also unique in the set of ACD compounds and no descriptor for the reactive epoxy group was introduced. If we equate this missing descriptor to the value for ICONJ (similar nucleophilic reactivity), then glycidic benzoate would be correctly predicted. Hydroquinone, a strong ACD chemical, has the opposite problem and was overpredicted. It is a statistical outlier because its positive value lies beyond the distributional range of the active class.

The equation shows satisfactory statistical strength as each of the nine descriptors is supported by five compounds. As the set increases in size toward n=100 (50 per class), we expect the number of required descriptors to increase. This expectation comes from a very large study of the Draize test by Enslein and coworkers[16]. For n=200-400, the number of descriptors required for discriminant analysis was k=17-36. Due to the care with which our sets are selected, we do not expect the descriptors to double or triple but simply to increase reasonably as new substructures are introduced.

Finally, we need to address the indeterminate range from 0.3-0.7 as defined by Enslein[16]. Compounds of borderline strength or uncertain model prediction will fall in this range. In the present case, ethylenethiourea is the most negative ACD compound (0.477) and methyl 2-aminobenzoate is the most positive non-allergenic (0.547). While both are incorrectly classified, neither is an outlier within the model statistics. This is nicely visualized by a residual plot of Y(estimate) versus actual class, clearly showing the indeterminate range.

Model Validation

Ten ACD compounds not included in the model were used for validation. This included three compounds known to be borderline in response as well as compounds of both simpler molecular structure (formaldehyde) and more complex (griseofulvin, penicillin G) than those used to derive the model. The results are shown in Table 1. The notation, GPMT refers to the guinea-pig

TABLE 1

Ten ACD compounds not included in the model.

Compound	Score	Class	Rated
Aniline	0.482	(0)	GPMT/90X(+/-)
Griseofulvin	0.566	(1)	2/Z
Formaldehyde	0.597	(1)	2/Z(+)
Picryl Chloride	0.770	1	3/Z(+)
Phthalic Anhydride	0.956	1	GPMT/90X(+)
m-Aminophenol	1.094	1	GPMT/100X(+)
Penicillin G	1.106	1	3/Z(+)
Piperonal	1.247	1	2/Z
p-Benzoquinone	1.613	1	GPMT/100X(+)
n-Propyl Gallate	1.820	1	GPMT/100X(+)

maximization test[4], 2/Z and 3/Z refer to moderate and strong ACD response (INPRET DB)[8] and (+) or (+/-) refer to the Kimber local lymph node assay[5,17]. Seven of the ten are correctly and strongly predicted to be Class 1 ACD irritants. The other three fall in the indeterminate range, 0.3-0.7, and require some discussion. Aniline is incorrectly predicted (score= 0.482) and is classed as moderate in the GPMT and borderline in the local lymph node assay. Griseofulvin is rated moderate in the GPMT but is known to be borderline in medical practice. It can be applied topically for ringworm in most people but may require oral administration for some. Formaldehyde has 20 entries in the INPRET database ranging from innocuous to

strong depending on the investigator and method of challenge.

All factors considered, these ten compounds are correctly predicted by the regression model, including those expected to be borderline.

CONCLUSIONS

As a first step toward a predictive model for ACD, this study was exceptionally fruitful. The derived relationship separating allergens from cosmetically innocuous chemicals provides both new and confirmatory evidence for the mechanistic nature of allergic contact dermatitis. The dichotomous descriptors for hapten and prohapten substructures confirm the reactivity and metabolic activation processes so ably described by Dupuis and Benezra[6]. Unique outliers like glycidyl benzoate suggest that more such descriptors will be required as we expand the structural scope of the study. The current goal is to double the data set to 50 allergens and 50 non-allergens. The candidate selection process to accomplish this is now underway.

Of greatest importance was the quantifying of site delivery in terms of calculable mechanistic factors. Calculation from molecular structure provides a means of estimating the behavior of an untested or conceptual chemical or drug. Basing the factors on properties related to known mechanisms gives us actual insight at the molecular level. Thus, the retarding effect of hydrogen bonding and lipophilicity are clearly consistent with preconceived mechanisms considered to be probable. In fact, these descriptors were selected for the purpose of probing the operation of such events. Offsetting the retarding effects of stratum corneum binding and lipophilic partitioning is the unexpected discovery of a bulk effect in promoting ACD response. While not anticipated, this effect is consistent with the vigour of the immune system response. The energetics of antigen recognition and lymphocyte templating will clearly depend on the size of the binding surface. Overall, these insights bring ACD response clearly into the same arena with drug delivery and percutaneous absorption.

The performance of the model in the validation tests is much better than expected for this intermediate stage of development. It was surprising that formaldehyde, griseofulvin and penicillin G were so well predicted as all are clearly outside the structural scope of the model, representing extrapolations rather than interpolations. It was a particular surprise that the three borderline chemicals were correctly predicted as such. This provides us with some hope for a future model to define mechanistic differences between weak and strong allergens. For the present, however, we are content to separate allergens from non-allergens.

Special Dedication: This paper is dedicated to the memory of our friend and colleague, Claude Benezra, who did so much to stimulate research in this field.

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PHILIP S. MAGEE

Dr. Magee is President of BIOSAR Research Project, an independent research and consulting company founded in 1983. His research enters on structure-activity problems related to living systems and the study of molecular mechanisms.

After completing the PhD in Physical Organic Chemistry (UCLA, 1955) he joined the Exploratory Chemicals Division of Chevron Research Company. Studies on new surface active agents, monomers, flame retardants, catalytic hydrogenation and oxidation represent this period.

In 1962, Dr. Magee transferred to the Ortho Division of Chevron Chemical Company to conduct research on new agricultural biocides. During the next 21 years, he synthesized over 3000 compounds for agricultural testing and supervised a major portion of the synthesis program. Two of these compounds are commercial insecticides of worldwide importance, methamidophos (MONITOR) and acephate (ORTHEME). In 1980, he was appointed Research Scientist, a unique position that he held until leaving to form his own organization.

Concurrent with his industrial research, Dr. Magee lectured extensively, edited books, and contributed chapters and papers on bio-mechanism and new approaches to synthesis of bioactive compounds. Four graduate level courses were presented at the University of California (Berkeley) in the period, 1956-1978. He is currently a Research Dermatologist at University of California School of Medicine (San Francisco) and a Courtesy Professor in the College of Pharmacy at Oregon State University (Corvallis). He chairs the International QSAR Society and serves on the editorial board of the European Journal, Quantitative Structure-Activity Relations.

Session II: Plenary Session

Co-Chairs: Drs. Neil Wilcox and Keith Booman

NEIL L. WILCOX, D.V.M., M.P.H.

Dr. Neil L. Wilcox pursued his undergraduate and graduate training in veterinary medicine at Michigan Statue University, East Lansing Michigan commencing in 1963 and received his D.V.M. in 1971. He practiced companion animal medicine for 3 years in Farmington, Michigan and for 15 years in Marshall, Michigan. While practicing veterinary medicine, Dr. Wilcox was active in several professional and community organizations. In addition to veterinary medicine, Dr. Wilcox's interests included health care policy and administration and served for 12 years on the Board of Trustees for Oaklawn Hospital, an acute care, 77 bed community hospital. His contributions to the hospital included Chairman, Long-Range Planning Committee, President, Oaklawn Hospital Board of Trustees, and President, Oaklawn Health Services.

Dr. Wilcox received a Masters of Public Health in Policy and Administration (M.P.H.) from the School of Public Health, University of Michigan, Ann Arbor, Michigan in 1989. He joined the FDA in March 1990 as a veterinary medical officer in the Division of Therapeutic Drugs for Non-Food Animals, Center for Veterinary Medicine (CVM) and was appointed Director, Office of Animal Care and Use in December 1990.

As representative of the Food and Drug Administration on animal welfare issues, Dr. Wilcox serves on several agency and inter-agency committees and is currently Chairman of the Inter-Agency Regulatory Alternative Group (IRAG). He is a member of the American Veterinary Medical Association and the American Public Health Association.

KEITH A. BOOMAN, Ph.D.

Dr. Booman has a Ph.D. in Organic Chemistry from the California Institute of Technology.

He was employed for 15 years by the Rohm & Haas Company and held research and supervisory positions in analytical chemistry and the development and environmental evaluation of detergent ingredients. He was Technical Director of the Soap and Detergent Association for 20 years, with responsibility for the Association's research on environmental and human safety, and for interaction with regulatory agencies on technical aspects. He was directly involved in the evaluation of alternative eye irritancy test methods for the last 10 years. Dr. Booman's publications have dealt with all the areas mentioned but in recent years have focused on the statistical analysis of environmental and safety data.

Now retired from the Association, Dr. Booman is a statistical analysis consultant. He is a member of the American Statistical Association and the Institute of Mathematical Statistics.

THE EVOLUTION OF *IN VITRO* TOXICOLOGY IN THE PHARMACEUTICAL INDUSTRY

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ABSTRACT

The decade of the 80's was marked by significant interest and growth in the application of biochemical and *in vitro* techniques in drug safety assessment. Within the safety assessment process, *in vitro* methods can contribute at the early stages of compound identification and selection as well as assisting in the resolution of safety issues arising in the preclinical or clinical phases of drug development. Knowledge of the basic mechanisms of toxicity disclosed by *in vitro* technologies can also play an important role in designing agents with improved safety profiles. While the current utility and value of *in vitro* techniques is evident, the further development and integration of *in vitro* techniques in toxicology will depend upon our ability to successfully meet existing scientific challenges. The future rests in bridging the gaps in our knowledge of toxicologic responses *in vitro* and *in vivo*, and designing model systems that offer confidence that these responses correlate biochemically and mechanistically.

I. INTRODUCTION

In vitro toxicology describes a field of endeavor which simply applies technologies inclusive of isolated organs, isolated tissues, cell culture, biochemistry and chemistry to the study of toxic or adverse reactions of xenobiotics. The objective of this presentation is to provide a perspective of

the evolution of in vitro toxicology, particularly as it applies to the pharmaceutical industry.

A. Historical Perspective

The decade of the 80's was marked by prominent interest and activity in the applications of in vitro techniques in toxicology, both in the academic and industrial sectors. This activity was manifested by the appearance of a number of journals devoted to in vitro, biochemical or molecular approaches to toxicology, the establishment of laboratories focused on the search for alternatives, and the conduct of numerous symposia and workshops devoted to the topic of in vitro toxicology. Most importantly, the past decade was associated with a growing commitment of industry to in vitro test development. This commitment was reflected by the formation of groups within many industrial safety assessment components which were focused upon investigational research often involving in vitro techniques. Several factors have impacted on these parallel developments in an additive fashion. First, the availability of the applied in vitro technologies themselves (e.g. cell culture, receptor pharmacology), and the personnel trained to perform them, blossomed in the 70's and 80's. Secondly, the contribution of the public sector in the developments of the 80's must also be recognized. Toxicology, particularly as an industrial activity, has always been strongly influenced by public opinion and pressure. The impetus for the formation of the Food and Drug Administration (FDA), and the issuance of regulatory laws commencing with the Food, Drug and Cosmetic Act of 1938, can largely be attributed to public reaction to tragedies such as sulfanilamide in 1937 and thalidomide in 1961. So too has public pressure for animal welfare concerns in the last decade played a significant role in solidifying the commitment to investigate and utilize in vitro techniques whenever possible.

Lastly, in viewing the progression of in vitro toxicology in the pharmaceutical industry, it is relevant to note that the advent of toxicology itself as a profession is a recent development in this century. This development can be reflected by the formation of the Society of Toxicology in 1961, and the establishment of drug safety units distinct from pharmacology departments within the industry in the 50's and 60's. Toxicology can therefore be regarded as a relatively

young profession when compared to pharmacology, whose professional society (ASPET) was established in 1908. Industrial laboratories have, therefore, been in the forefront in the incorporation of *in vitro* techniques in toxicology.

II. INDUSTRIAL APPLICATIONS

The number and types of *in vitro* toxicologic models utilized in the pharmaceutical industry encompass virtually every major target organ of toxicologic interest (e.g. liver, kidney, heart, brain, skin, eye). The breadth of the systems available is impressive, and again signifies a relatively rapid progression of *in vitro* test development in toxicology.

A. Philosophical and Scientific Considerations

The fact that many of the *in vitro* test systems are utilized in toxicology underscores the commitment of industry to the principles of reduction, refinement and replacement of whole animal *in vivo* tests whenever possible. However, industrial toxicologists must appropriately balance this commitment with a fourth "R", responsibility. The ethical and legal responsibility of the toxicologist is to assess the safety of new products, and to protect, to the best of his or her ability, the public from harm. Thus, current test procedures cannot be abandoned unless the new tools can be adopted with the assurance that adverse properties will be reliably detected. A key factor in the application of *in vitro* techniques in toxicology involves the degree of correlation between events occurring *in vitro* and those which the toxicologist evaluates in the intact animal. This correlation determines the ultimate scientific value of the techniques, and the level of confidence associated with a particular test in terms of its predictability from a safety perspective.

The criteria that determine the degree of correlation or level of confidence in a given test are summarized in Table 1. The first of these is predictability, both qualitatively and quantitatively. Qualitatively, do the rankings or order of toxicities *in vitro* correlate with the order of toxicity *in vivo*? From a quantitative aspect, what are the dose-response characteristics from which potency estimates and comparisons can be made? Finally, how do drug concentrations *in vitro* compare with those achieved *in vivo*? The second criteria to

examine is that of test system identity. To what degree does the *in vitro* system structurally and functionally mimic the *in vivo* organ? Thirdly, the area of mechanisms of cellular injury is a key criteria to consider in the utilization of *in vitro* models for toxicologic evaluation. The variety of mechanisms that can play a central role in cell injury underscores the need to recognize specifically how the response of the *in vitro* test system correlates with the response in the intact organ or organism. Lastly, the topic of compensatory factors needs to be considered. How does the ability of the *in vitro* system to scavenge toxic reaction products compare with *in vivo* abilities? What detoxification or toxification pathways relevant to the *in vivo* fate of toxins/chemicals are present in the *in vitro* test system? These questions must be addressed prior to utilization of *in vitro* procedures in order to establish a clear understanding of the assumptions and/or limitations of the data to be generated. While *in vitro* systems need not fulfill all the correlative criteria outlined to be useful, the successful achievement of these criteria will be required to replace current whole animal tests.

TABLE 1

CRITERIA FOR ESTABLISHING IN VITRO-IN VIVO CORRELATIONS

PREDICTABILITY
- Qualitative: rankings (% maximum response)
- Quantitative: dose-response
  relative drug concentrations *in vitro*/*in vivo*

IDENTITY
- Structure: morphologic correlates
- Function: tissue specificity
  e.g. transport characteristics, metabolic pathways

MECHANISMS OF INJURY
- Membrane damage (structural, functional)
- Synthetic activity (protein, RNA, DNA)
- Metabolic poisoning (0_2 utilization/consumption, glycolysis, gluconeogenesis)

COMPENSATORY FACTORS
- Biochemical scavengers (glutathione, metallo-proteins)
- Detoxification pathways
- Toxification pathways --oxidation/reduction/hydrolysis/conjugation

B. The Role of In Vitro Tests in Toxicological Testing

An examination of the role that in vitro tests play in toxicology requires a preliminary discussion of the safety assessment process itself. Preclinical toxicologic assessment involves a series of decisions at the pre-project and product safety stages of development. Although the vast majority of in vitro techniques have not been fully validated to meet all of the in vitro - in vivo criteria discussed above, many can still play an important role within the toxicologic tier structure. At the pre-project evaluation stage, several chemical analogs of a particular pharmacological series may be under consideration for development. In vitro techniques are particularly powerful at this level of decision-making as product candidates (or project team compounds) are identified. Specifically, in vitro tests facilitate the evaluation of agents for specific target organ effects (e.g. hepatotoxicity, mephrotoxicity), contributing directly to the product selection process. Such preliminary evaluation and screening may greatly enhance the probability of success of new agents in subsequent safety testing in animals and man. Following selection of a compound for further development, a safety assessment "package" is conducted prior to clinical introduction of the new agent. At this stage, in vitro techniques are also applied in performing specialized evaluations (e.g. genotoxicity, neuromuscular transmission) as well as in resolving issues that may have arisen in in vivo safety studies. These in vitro data are submitted to regulatory agencies as part of the overall safety assessment of a new chemical entity. In vitro techniques are also useful in problem-solving activities such as exploring the mechanisms of target organ toxicity or performing species comparisons in vitro. Similarly, at the clinical stages of drug testing, safety issues may be most appropriately explored through the use of in vitro techniques to study mechanisms of an adverse finding; i.e. to compare species including man.

It should also be noted that in vitro techniques permit the toxicologist to participate in the drug discovery process itself. Since the development of safe, as well as effective, products is the ultimate goal of the pharmaceutical industry, the role that in vitro models can play in the design of new agents and improvement of existing ones is particularly exciting. Specifically, in vitro systems permit toxicologists, as well as biochemists and chemists, to explore structure-activity relationships involved in toxic reactions.

The potential to define structural features associated with toxic reactions, and to determine if those characteristics are distinct from those required for the desired therapeutic activity, represents an integral role the toxicologist can play in the drug discovery process. With respect to existing products, the modeling of toxic reactions *in vitro*, particularly at the mechanistic level, provides avenues for designing strategies to ameliorate or prevent injury at the target organ level.

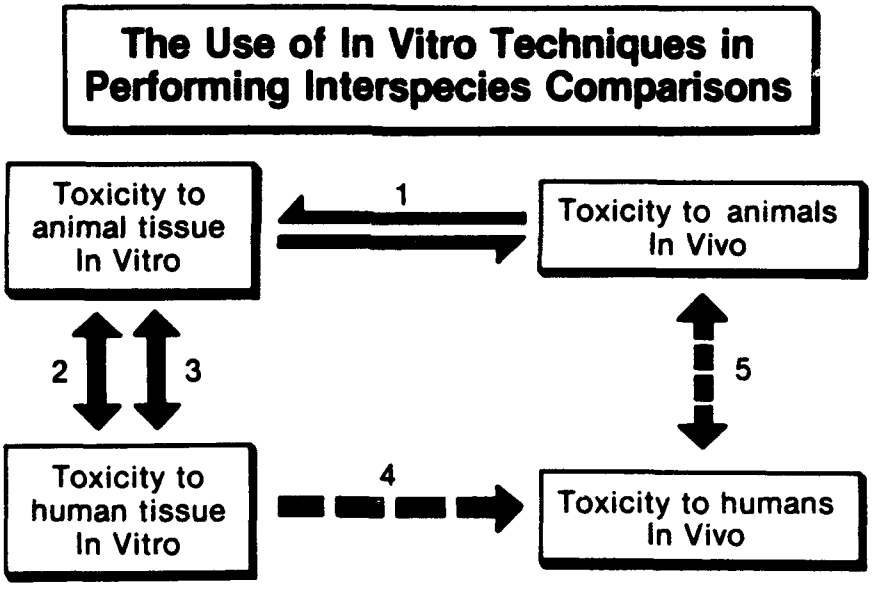
An example of a simple *in vitro* model which has contributed in multiple ways to the safety assessment process involves the use of isolated renal membranes to study the nephrotoxicity of aminoglycoside antibiotics. By utilizing isolated renal brush border membranes, mechanistic information and structure-activity relationships were obtained by examining the binding of radiolabelled aminoglycosides (e.g. gentamicin) to the membranes *in vitro*[1,2]. In addition, inhibitors of aminoglycoside nephrotoxicity *in vivo* were identified utilizing the isolated renal membrane model[3], contributing directly to product improvement and design.

III. THE FUTURE

The future of *in vitro* techniques in toxicologic assessment takes us back to our earlier discussion of the philosophical and scientific considerations operating in the evolution of alternative methods.

Scientifically, the future will depend on the level of confidence achieved that *in vitro* systems provide information that is representative of the *in vivo* processes the toxicologist seeks to model and predict. The degree to which this level of confidence will evolve is directly proportional to the fulfillment of the scientific criteria outlined in Table 1 which determine the strength of the *in vitro* - *in vivo* correlations obtained. Because gaps remain in our knowledge with respect to these important criteria, the future will also depend on advancements in available knowledge and technology with respect to biochemical or *in vitro* toxicologic events. Specifically, challenges for the future involve the continued development of current *in vitro* models to provide physiological and morphological characteristics which correlate more closely with target organs *in vivo*. These challenges will include defining media constituents that will facilitate the retention of normal morphology and metabolizing capabilities *in vitro* as well as the establishment of systems

that reproduce the dynamic features of organs *in vivo*. With *in vitro* - *in vivo* correlations playing a key role in *in vitro* test development, the growth of technologies that focus upon differentiated functions, cellular relationships, human models, and other *in vivo* properties, such as fluid dynamics, will be critical to the future applications of *in vitro* techniques in toxicology. As predictability and correlation of *in vitro* models with specific *in vivo* events is achieved, toxicologists can look forward to performing meaningful species comparisons *in vitro*, involving direct preclinical assessments in man (Figure 1).



1=Correlation of In Vitro with In Vivo Observations
2=Development of Parallel In Vitro Systems in Man
3=Comparison of In Vitro Sensitivity of Different Species
4=Potential Toxicity In Vivo
5=Comparison of In Vivo Sensitivity of Different Species

CONCLUSION

In summary, an examination of the current state-of-the-art in the pharmaceutical industry illustrates exciting and important role(s) that *in vitro* systems can play in toxicologic assessments. With the development of *in vitro* models more closely representing organ function and dynamics, the ability to accurately predict toxic reactions *in vivo* will be enhanced. As time and experience build scientific confidence and validation of these *in vitro* models, one can envision the toxicologist utilizing *in vitro* models more extensively in the assessment of toxicity. However, in order to advance alternative methodology to this stage, it is clear that the toxicologist must continue to take an active role in the research of toxic mechanisms and the development of the tools to study them. Without such a commitment, the field of *in vitro* toxicology might be expected to remain static. Thus, while the current uses of *in vitro* techniques have significantly reduced animal use, and advanced our understanding of toxic mechanisms, the full potential of *in vitro* models to further reduce, refine and replace whole animal tests has certainly not been realized. The future, in fact, is only limited by the amount of energy and creativity as scientists we can apply to *in vitro* test development.

For the present, the utilization of *in vivo* toxicologic models is imperative for responsible risk assessment of new chemical entities. At the same time, the use of the many *in vitro* models currently available can serve as valuable adjuncts to these *in vivo* assessments, not only reducing the number of animals used in risk assessment, but providing unique information and possibilities for scientists involved in the drug discovery and development process.

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REVIEW AND EVALUATION OF IRAG EYE IRRITATION WORKSHOP

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Staff from 3 federal regulatory agencies presented proposals for modifying the Draize eye test. They include the use of screens to truncate the need for any animal testing (structure-activity relationships, pH, *in vitro* test results and acute dermal toxicity and irritation). Currently agencies require placing a given volume of test material into the eye of 6 rabbits. Proposals incorporate the use of topical anesthetics and the dosing of 2 or 3 animals in most cases and a lower volume in 1 animal for suspected severe irritants. Treated animals are scored for corneal and iritic endpoints and conjunctival redness. Animals demonstrating any adverse effect at any scoring time are designated positive, and 2 positive animals define a positive test. A questionnaire was used to evaluate workshop participants' reactions. Development of definitive agency positions and exploration of international harmonization are anticipated.

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REGULATORY REQUIREMENTS FOR VALIDATION OF *IN VITRO* ALTERNATIVE TESTS

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Abstract

When developing *in vitro* alternative tests, one must consider whether the alternative assay is to be used as a screen or as a replacement for the traditional eye test and whether the alternative assay will provide the same information that the animal assay provides. Criteria for *in vitro* tests used as screens or replacements should include a rationale for their use instead of other available tests; the biological or physiopathological relevance of the *in vitro* endpoint to the effect produced *in vivo*; and intralaboratory as well as interlaboratory testing to ensure reproducibility.

The U.S. Food and Drug Administration cannot precisely prescribe what is required for validation of a test or method, because there are a variety of purposes for which tests are used. However, there are some criteria that must be met before an alternative test can be accepted as valid by the general scientific community and used for regulatory decisions (1).

When developing an alternative test, one must determine whether the alternative assay is to be used as a screen or as a replacement for the animal test. An assay used as a screen will require less stringent acceptance criteria than are required for a replacement test. A screen is a preliminary test for the assessment of a toxicologic effect. It is used for making preliminary decisions or establishing the direction for further

testing. Screens answer fewer and less complex questions than do replacement tests, and the results from screens must be confirmed by more definitive testing. A replacement test, however, must provide the same answers as in vivo methods for the assessment of toxicity. It must provide data that allow a definitive assessment of toxicity.

One must also have a thorough knowledge of the in vivo assay to be replaced. This knowledge should include the test procedures and the regulatory information the test provides (e.g., eye irritation responses of the cornea, conjunctiva, and the iris, and the methods of scoring). In certain instances, such as for eye irritation responses, one must also be familiar with how these data are used to classify test substances.

An investigator should clearly state the rationale for recommending the use of a particular in vitro test in relation to the other available tests. A few alternatives to the use of live animals for the eye irritation test include the use of chick chorioallantoic membrane (2), agarose diffusion (3), Eytex (Ropak, Irvine, CA) (4), uridine uptake inhibition (5), neutral red uptake (5), rabbit corneal cells (6), and the Tetrahymena motility assay (7). Each test has its own strengths and weaknesses, and new tests must contribute additional needed information or be much improved (e.g., easier to use or less expensive) over the more traditional methods that are already available. The endpoint for the in vitro test should be similar to the in vivo endpoint. For example, if the in vivo assay provides information about ocular irritation, the endpoint of the alternative test should also address ocular irritation.

Occasionally validation studies focus on the correlation between positive and negative results in the new assay compared with the animal method, whereas the relevance of the endpoint is not addressed. A good correlation between test results for the alternative and animal assays should not be the major criterion for assessing the validity of alternative tests.

For example, the human embryonic palatal mesenchyme (HEMP) cell line has been suggested as a screen for teratogens. The endpoint for this test is inhibition of cellular proliferation. Cellular proliferation is essential to normal development and its interruption could lead to depressed growth and possibly to teratogenesis. However, how would a cytotoxic agent be differentiated from a teratogen? The correlation between known teratogens and nonteratogens in the assay must be established, but without the basic scientific foundation underlying the endpoint, the conclusions reached are suspect. It is far simpler to determine and express correlation in terms of an endpoint, e.g., to say, "this teratogen inhibits cellular proliferation" than to state "because this agent inhibits cellular proliferation it is a suspected teratogen." The latter statement is more

scientifically sound, because it implies some relationship between the endpoint in the alternative assay and the effect that is found in the animal. Correlations are only as good as the biologic or physiologic endpoint. How relevant the endpoint is to the effect being measured strengthens or weakens the interpretations and conclusions drawn from such an alternative assay and, consequently, its regulatory usefulness.

Validation studies can be undertaken only after a biologically or physiologically relevant endpoint has been established and a standardized protocol has been developed. As the validation studies proceed, the protocol must be modified to enable identification of all agents that are to be tested. Validation, therefore, is a dynamic process and cannot be described by a rigid set of guidelines or rules.

Once the protocol is developed, the next step is to conduct intralaboratory and interlaboratory testing. These investigations should include as many agents and laboratories as practically possible, given the type and quantity of information the test will provide.

Qualitative data, which provide information as to whether the assay responds in the same manner as the more traditional assays, are also needed. A broad spectrum of chemical classes must be tested with the new assay before any correlation between results from the alternative and traditional tests can be objectively evaluated.

Quantitative data, which could involve determining whether the rank order of positive agents in terms of toxicity or other measured effect is the same or similar in both assays, are also needed. Should an alternative test closely match the animal test it is to replace in predicting which agents are the most and least hazardous? If there are wide and disparate results, how much confidence can be placed in the alterative test? These questions must be addressed before these new technologies are used.

An <u>in vitro</u> alternative test should not be discarded simply because it does not function with universal perfection. Indeed, it is unlikely that any single alternative will be completely acceptable in all circumstances. For example, some tests may be highly effective only for water-soluble materials. Others may be effective for solids, granular substances, or pastes. It is likely that certain types of products may prove amenable to particular tests, while other products may need a different battery of tests. For instance, it may be possible to develop tests that reliably indicate the eye irritation potential of surfactants in soaps and detergents. However, these tests may be less useful for assessing the irritation potential of halogenated organic solvents used in dry cleaning and spot removers. The critical consideration is that the usefulness of any test depends

on the understanding of the capabilities and limitations of the *in vitro* test for the types of materials to be tested.

When one is selecting the chemicals to be used for validation studies, John Frazier (8) suggests the following. The test substances used should include chemicals from all the general classes of toxicity (nontoxic, mildly toxic, and highly toxic) and from all defined chemical classes (so structure-activity relationships can be developed). Some of the chemicals used should provide data on mixture interactions. And *in vivo* data on each of the chemicals should be provided so that the test results can be compared between assays.

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REGULATORY REQUIREMENTS FOR VALIDATION OF *IN VITRO* ALTERNATIVES

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Two factors need to be considered initially when developing an alternative test. The first is to determine whether the alternative assay is to be used as a screen or as a replacement for the traditional eye test. An assay used as a screen will require less stringent acceptance criteria, for it is designed to answer fewer and less complex questions, e.g. only potential eye irritancy is assessed. An assay used as a replacement will be used to establish hazard or lack thereof (safety). In other words, to clearly state that a chemical product is an eye irritant. Establishing hazard or lack thereof is a much more intricate process and must be accomplished with the best science available.

Secondly, to develop an alternative assay, one should have knowledge and/or experience with the *in vivo* assay intended to be replaced. This knowledge should be in terms of, not only the procedural aspects of the test but also the regulatory information it provides, e.g., responses, scoring and their use in classification. Thorough consideration of the regulatory information is very critical for a test intended to be used as a replacement.

The following may be considered as criteria for *in vitro* tests used as screens or as replacements for the eye irritation test in rabbits:

1. Rationale: There should be a clear statement regarding the rationale for the use of a particular test in relation to the availability of other tests.

2. Relevance: There should be biological or physio-pathological relevance of the *in vitro* endpoint to the effect to be detected *in vivo*.

3. Validation: Intralaboratory as well as interlaboratory validation must be conducted.
   a. Requires the development of a standardized protocol.
   b. Requires intralaboratory and interlaboratory reproducibility of results from the standard protocol.

Qualitative aspect; provides information as to whether the assay responds in the same manner as the traditional assay (predictability).

Quantitative aspect: ensures that the degree of severity or intensity of the effect as measured by the alternative test is the same as that measured by the traditional assay.

Chemicals considered to be representative of the defined category of interest should be evaluated.

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CONSUMER SAFETY, HARMONIZATION OF TEST METHODS AND CLASSIFICATION SYSTEMS, AND VALIDATION OF ALTERNATIVES

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The needs of consumer safety should not be overwhelmed by the concerns about trade and animal welfare during the international harmonization of test methods, classification systems, and communication systems currently in progress. The harmonization of test methods and classification systems should facilitate and may even accelerate development and validation of alternative test methods. The current role of Organization for Economic Development and Cooperation, International Labour Office, and other United Nations organizations in this process is briefly presented.

The consumer population includes susceptible individuals such as children, the elderly, and the infirm, while the worker population involved in manufacture, transport and distribution, generally includes healthy adults. The workers are generally exposed to a few hazardous substances whereas the consumer can be exposed to multiple hazardous substances that are present in and around residences, transportation and public places. Exposure of multiple hazardous substances to a susceptible population may pose a greater risk of injury. For these and other reasons, consumer safety has always been given special consideration in the regulatory process. It is crucial that this special consideration continue during the current efforts to harmonize test methods and classification systems. Concerns about trade and animal welfare should not overwhelm concerns about consumer safety.

International harmonization of test procedures, classification systems, and communication systems for hazards to humans and the environment should improve protection of humans and environment, facilitate international trade, and reduce the number of animals used in testing. International acceptance of test results from harmonized test protocols that are designed to generate data suitable for use in a harmonized classification system will reduce the number of tests currently conducted to meet various national and regional classification systems. The harmonization of test protocols and classification systems should also facilitate

and possibly accelerate the development and acceptance of alternative test methods since the alternative methods need to meet the requirements of a harmonized system rather than multiple national and regional systems. However, the interlaboratory validation phase of an alternative test(s) will need to include laboratories in different countries. Involvement of an appropriate harmonizing organization early in the validation process should facilitate international interlaboratory validation and adoption of alternative methods. However, during the development and validation of alternative methods one should assure that the alternative methods generate data suitable for the evaluation of risk of injury, with an adequate margin of safety for consumers, and especially for susceptible populations.

The Organization for Economic Cooperation and Development (OECD) through its Chemicals Group and Management Committee has been involved in the development of test protocols for hazards to man and environment posed by chemicals. The data generated using these protocols is accepted by the member nations. The membership of OECD includes most of the industrialized nations. These include United States, Canada, Germany, Australia, Austria, Belgium, Denmark, Spain, Finland, France, Greece, Island, Italy, Japan, Luxembourg, Norway, New Zealand, Netherlands, Portugal, United Kingdom, Sweden, Switzerland, and European Economic Community. The OECD will continue to be the primary forum for the harmonization of test methods.

At the United Nations Conference on Environment and Development (UNCED, June 3-13, 1992) held in Rio de Janeiro, Brazil, it was agreed to develop an internationally harmonized system for testing, classification and communication of hazards posed by chemicals[2]. This responsibility was assigned to The International Programme for Chemical Safety (IPCS). The IPCS is composed of World Health Organization (WHO), International Labour Office (ILO), and United Nations Environment Programme (UNEP). The IPCS has formed a Coordinating Group for the Harmonisation of Chemical Classification Systems (IPCS/CG.HCCS). This group has drafted a Terms of Reference and Work Programme[3] and has decided that the focal point for the harmonization of physical hazards posed by chemicals will be ILO and focal point for the harmonization for all health effects will be OECD.

[1] The opinions expressed in this article are those of the author and do not necessarily represent the views of the Commission.

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CONSUMER SAFETY, HARMONIZATION OF TEST METHODS AND CLASSIFICATION SYSTEMS AND VALIDATION OF ALTERNATIVES

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The consumer population includes susceptible individuals such as children, the elderly, and the infirm. Consumer exposure to multiple hazardous substances can occur, which may cause of greater risk of injury. For these and other reasons, consumer safety has always been given special consideration in the regulatory process. It is crucial that this special consideration continue during the current efforts to harmonize test methods and classification systems. Concerns about trade and animal welfare should not overwhelm concerns about consumer safety.

During the validation of new test methods, assurances must be made that alternative methods generate data suitable for the evaluation of risk of injury, with an adequate margin of safety for consumers, and especially for susceptible populations. The international harmonization process simplifies the development and validation of alternative methods since the generated data need to meet criteria of a harmonized test and classification system. The interlaboratory validation phase needs to include laboratories in different countries. Early involvement of a harmonizing organization will facilitate international interlaboratory validation and adoption of alternative methods.

The International Programme for Chemical Safety will be the primary forum for the harmonization of classification systems, except for the acute oral toxicity and environmental hazards, which will be harmonized by the Organization for Economic Cooperation and Development (OECD). The OECD will be the primary forum for the harmonization of test methods.

*The opinions expressed in this article are those of the author and do not necessarily represent the views of the Commission.

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Dr. Gupta received a degree in veterinary medicine from the University of Rajasthan, India, and a Ph.D. in anatomy from the University of Southern California. He received post doctoral training in veterinary surgery, veterinary pathology, cryobiology, pharmacology, physiology and tissue culture. He taught neurosciences to medical students for 5 years before joining the Consumer Product Safety Commission. At the Commission he functions as a veterinarian and a toxicologist. He was one of the organizers of the joint government and industry workshop on Alternatives to the Draize Eye Test in 1988.

Session III: *In vitro* and Other Alternatives in Ocular Toxicology

Co-Chairs: Van M. Seabaugh and Alan C. Katz

VAN M. SEABAUGH, M.S.

Mr. Van Seabaugh began working for the U.S. Government in 1958 as a pharmacologist/toxicologist with the Department of Defense. In 1973, Mr. Seabaugh joined the Consumer Product Safety Commission following a 10 year association with the Food and Drug Administration. He is currently employed by the U.S. Environmental Protection Agency. Mr. Seabaugh is a member and former president (1989-1991) of the Society of Comparative Ophthalmology. He is a full member of and advisor to the Society of Toxicology and a charter member of the American College of Toxicology. He belongs to the American Industrial Hygiene Association and is on the editorial boards of several professional journals. Mr. Seabaugh is an active participant on various national and international scientific committees.

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Alan Katz is President of the Society of Comparative Ophthalmology and Executive Director of Toxicology and Analytical Chemistry for Technical Assessment Systems, Inc. He has approximately 25 years combined experience in the chemical and pharmaceutical field, including 13 years of industrial experience in pharmacology and toxicology. His responsibilities with TAS include project administration and supervision of multi-disciplinary staff to provide analyses and evaluations of toxicological and residue chemistry issues related to study design, protocol review, data interpretation and regulatory compliance.

As a senior toxicologist with the Environmental Protection Agency, he was responsible for auditing and evaluating laboratory data supporting pesticide registrations for numerous chemicals. He also prepared comprehensive toxicology chapters for several Pesticide Registration Standards and served as an expert on ocular toxicity issues in the Office of Pesticide Programs. In 1985, he drafted guidelines for special studies to determine the systemic ocular toxicity potential for acute and chronic exposure to organophosphates. Previous employers include Stauffer Chemical Company, Johnson & Johnson (Ortho Pharmaceutical Corporation and Johnson & Johnson Research Foundation), Cooper Laboratories, Rockefeller University and Union Carbide Corporation. His expertise ranges from designing and managing acute, subchronic and chronic toxicity studies in accordance with GLPs and TSCA/FIFRA/OECD Guidelines and Regulations to interpreting the results of these studies and evaluating their significance.

Mr. Katz holds a B.S. in biology and a M.S. in human physiology from Fairleigh Dickinson University. He has been a Diplomate of the American Board Toxicology since 1980. He also holds a Certificate in Management from Central Connecticut State University. Mr. Katz is the recipient of the Environmental Protection Agency's award for his outstanding service to the Toxicology Branch, Hazard Evaluation Division of the Office of Pesticides and Toxic Substances.

HISTORICAL PERSPECTIVES

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Abstract

This brief introductory talk gives some background information, and briefly discusses some toxicology historical perspectives. From presentations at this session of the meeting [Current Concepts And Approaches On Animal Test Alternatives; Session (In Vitro And Other Alternatives In Ocular Toxicology; February 4,5,6 1992)], we will learn more about this subject of in vitro and other alternatives for testing.

Introduction

Almost thirty four years ago, I was a soldier scientist at this military instillation. I stayed on as a civilian for three more years before transferring to the U.S. Food & Drug Administration (FDA), the U.S. Consumer Product Safety Commission (CPSC), and I am presently with the U.S. Environmental Protection Agency (EPA).

At some point in their careers, I have discovered that many scientists were employed at the Aberdeen Proving Ground, MD. (Edgewood area). In my conversations with some of these people, we all agree upon is that it is an excellent place. When I was a soldier scientist at the Edgewood Arsenal, the building used for this symposium was the post theater. I could come to see the latest movie for twenty five cents. Little did I dream at that time that I would be attending this symposium and co-chairing this session today. It is a homecoming for me, and there is "no friend like an old friend." Over the years, I have kept in contact with Edgewood.

When I was at the CPSC, we had an interagency agreement with Edgewood's Bernie McNamara (Chief, Toxicology Division) for about 10 years to conduct toxicological tests. This gave me a chance to come back to Edgewood on many occasions. Times have changed with increased costs for movie tickets, and other commodities. We too have increased our knowledge for medical testing.

Discussion

When I think about testing procedures for eye irritation, I think of John Draize. He was also employed at this military post. Among other things, John's job was to study military chemicals for potential eye effects. The Draize protocol is similar to Friedenwald's, but John's main contribution to ocular testing was the scoring system. The Draize scoring system gave numerical equivalents for brief subjective statements for cornea, iris, and conjunctivae. John moved to the FDA in 1938. Later on, the FDA needed an eye test for the purposes of testing drugs and cosmetics, and John had the answers. John retired from the FDA in 1963, and Frank Marzulli left Edgewood to take over that vacated branch chief job. They worked together for one month before John's retirement. I had the opportunity to talk with John and review his many volumes of eye testing data.

What are the historical perspectives for toxicology? In 1986, I was involved with the planning of the Society of Toxicology's (SOT) 25th annual meeting. We were able to get some copies of old scientists' photographs from the National Library of Medicine to be displayed at the SOT meeting. The oldest photograph we used was that of Paracelsus. Paracelsus (1493-1541) was a Swiss physician who was a professor of medicine. In 1990, John Doull (Professor of Toxicology & Pharmacology; U. Of Kansas Medical School) gave his "Historical Perspectives in Toxicology" speech at a symposium entitled New Horizons In Molecular Toxicology. John said that perhaps some of us would say that primitive man conducted the first toxicological study when he fed some of his food to his dog to see if it was safe to eat. To add to this dialogue, a quick look in the library can give us a glimpse of the historical perspectives for animal use in research and testing.

From recorded history, the pig was the first animal to be used for medical research [Garland's work (1500)]. As early as the 1600s, medical research was being conducted on people. Galvandis (1600s) conducted medical research with humans. Rembrandt's paintings as early as 1632 depicted medical research being conducted on people. Some of the kings of European countries had professional tasters to see before hand if the food to be consumed by the king and his family would kill or make the tasters ill. Claude Bernard (1800-1860) wrote a book entitled, "An Introduction To The Study Of Experimental Medicine." From 1861-1889, history tells us about: 1) The studies of Louis Pasteur who studied chemistry, bacteriology, diseases of the silk worms, rabies, and anthrax. 2) Robert Koch and F.K. Clein who studied infectious diseases. They wanted to know what killed the small animals, and did it apply to humans? 3) The Russian physiologist (Pavlov) who studied

associated and conditioned responses. 4) Cooper Curtis who studied Texas fever in cattle.

In 1892, newspapers advertised that they would pay twenty-five cents each for dogs or cats to be used in medical research. In 1875, J.C. Dalton published a book entitled, "Experimenting On Animals." In 1982, James Turner published a book entitled, "Reckoning With The Beast," and the question is asked if we are the beasts? Around 1876: 1) Henry Byrd founded the first American society for anticruelty to animals (ASPCA, N.Y., N.Y.). 2) George T. Angell founded the Massachusetts anti-vivisectionist society. In 1890, an attempt was made to get Federal legislation for the treatment of animals. The legislation did not pass.

When I was working at the FDA in the 1960s, I saw pictures in FDA's museum of its Poison Squad. In 1904, FDA was still using human employees to test food preservatives (e.g., boric acid, salicylic acid, their derivatives and formaldehyde) for toxicity.

What has happened in recent years? From my own recollection, I will mention a few things. In 1959-60, the drug thalidomide changed the course for animal testing. The Society Of Toxicology (SOT) was founded in 1961. In 1963, the Public Health Services granted a policy statement entitled, "Guide For The Care And Use of Laboratory Animals. In 1966, the Animals' Welfare Act was created. I can remember a meeting in the 1970's held at the Pan American Building by the National Institutes of Health and others on the use of animals.

In 1973, the Society of Comparative Ophthalmology was founded, and from that time has been one of the leaders in educating the public on **in vitro** and **in vivo** ocular testing. In 1977, the American College of Toxicology was founded. In 1980, Peter Singer published, "Stop Slaughter Of The Innocent."

The Cosmetic Toiletry & Fragrance Association (CTFA) held workshops on the use of animals. One of them was held in 1980, and was entitled "Proceeding Of The CTFA Ocular Safety Testing Workshop: **In vitro** and In vivo Approaches." We know about the work done at the Rockerfeller University to help find ocular alternatives for animal testing. The Office of Technology Assessment, Congress of the United States published a book, "Alternatives To Animal Use In Research, Testing, And Education (1986)." The book discussed a range of options for congressional action for public policy regarding the use of animals. The Catholic University continues to conducted workshops on tissue and cell culture techniques for alterna-

tive testing. The Johns Hopkins University alternative testing program is an on going program. Other information has been presented at workshops, meetings, and publications on the issues of in vitro and in vivo ocular testing.

In closing, I give you my thoughts about the present status of in vitro and in vivo ocular and dermal irritation testing. These thoughts are perhaps best summarized in the position paper issued by the Society of Toxicology (**Fundamental And Applied Toxicology** 13, 621-623 (1989; Prepared by the Animals In Research Committee of The Society Of Toxicology and Approved by the SOT Council).

"...Acute eye and skin irritation tests on chemical substances are conducted in order to characterize the hazards associated with ocular or dermal exposure. At the present, tests in intact animals are the only means of assessing the potential hazard from such exposure other than directly in man. Although validated in vitro alternatives to eye and skin irritation tests in animals are not available currently, many tests under development show promise and may be useful as initial screening techniques. Complete validation of these alternate forms of testing for irritation may reduce the need to use whole animals. Until these procedures have been thoroughly tested and validated, the investigator will have to rely on conventional methods. In each case, however, attention should be given to design and conduct of the study to reduce the numbers of animals to minimize animal discomfort."

"...As part of ongoing test programs many investigators have also refined and modified existing in vivo techniques to include: 1. The use of prescreens to identify those materials that may be corrosive or severely irritating. This would include, for example, the practiced exclusion of materials with very acidic or alkaline properties (which would impart a high potential for irritation). In addition, knowledge of the outcome of the skin irritation test before the test for ocular irritation permits a decision on the utility of the latter test: it is reasonable to conclude that severe skin irritants will also be severe eye irritants without actual test data..."

"...There are currently more than 60 proposed tests for predicting eye irritation which do not employ intact animals (Fraizer et al., 1987)...None of these proposed models are yet validated or evaluated for a broad range of chemical moieties, and none can be relied upon to provide the scientific reliability or predictive accuracy which would be required of a new test for regulatory or legal acceptability. Many hold promise for this and may be suitable, at least, as screens as further validation studies are being conducted."

Overview of *in vitro* Ocular Irritation Test Systems and Other Evaluation Status

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ABSTRACT

Active programs to develop in vitro alternatives to the rabbit ocular irritation test have been underway for more than ten years, with more than 70 proposed test methods of six different classes being the result. Large collaborative multilaboratory "evaluation/validation" programs, as will be reviewed here, have been conducted for the last five years, revealing both strengths and weaknesses of these alternative tests. Yet neither in vitro alternatives nor significant alterations in the basic in vivo design have been accepted as replacements by regulatory agencies. This history and the extent major results of current work, are the key determinants limiting possible means of, and limitations on future advancements in this area.

The ocular irritancy test has been the most controversial animal test in toxicology from the standpoint of both the public perception and the humane use of animals. There has been some grounds for this concern, but testing practices have changed significantly since 1980. These changes have reduced (but not eliminated) grounds for public concern.

There have also been scientific grounds for reconsidering the appropriateness of the test as currently performed in rabbits, and these have further stimulated attempts to develop in vitro alternatives to the rabbit eye based test. The technical rationales for seeking alternatives can be summarized as follows:

1. Strict Draize scale testing in the rabbit assesses only three eye structures (conjunctiva, cornea, iris) and traditional rabbit eye irritancy tests do not assess cataracts, pain, discomfort, or clouding of the lens.
2. In vivo tests assess only inflammation and immediate structural alterations produced by irritants (not sensitizers, photoirritants, or photoallergens or route specific lethality).
3. Technician training and monitoring are critical (particularly due to the subjective nature of evaluation).
4. Rabbit eye tests do not perfectly predict results in humans, if our objective is either the total exclusion of irritants on an absolute basis (that is, without false positives or negatives) or the identification mild irritants. Some (such as Reinhardt et.al., 1985) have claimed that these tests are too sensitive for such uses.
5. There are structural and biochemical differences between rabbit and human eyes which make extrapolation from one to the other difficult. For example, Bowman's membrane is present and well developed in man (8-12 mm thick) but not in the rabbit, possibly giving the cornea greater protection.
6. Lack of standardization of protocols and test procedures.
7. Large biological variability between experimental units (ie. individual animals).
8. The available animal data are available as large, diverse and fragmented databases which are not readily available or comparable.

These have made ocular irritation the most fertile area of alternative test development. There are now more than 70 proposed in vitro test systems which can be considered in six different classes: morphology, cell toxicity, cell and tissue physiology, inflammation/immunity, recovery/repair, and miscellaneous. Some examples of tests in each of these categories are presented below in Table 1 (adapted from Frazier et. al., 1987).

TABLE 1: IN VITRO ALTERNATIVES FOR EYE IRRITATION TESTS

I. MORPHOLOGY

1. **Enucleated Superfused Rabbit Eye System (Burton et. al., 1981).**
2. **Balb/c 3T3 Cells/Morphological Assays (HTD) (Borenfreund and Puerner, 1984).**

II. CELL TOXICITY

1. **Adhesion/Cell Proliferation**

   a. **BHK Cells/Growth Inhibition (Reinhardt et. al., 1985).**
   b. **BHK Cells/Colony Formation Efficiency (Reinhardt et. al., 1985).**
   c. **BHK Cells/Cell Colony Forming Assay (North-Root et. al., 1982).**
   d. **SIRC Cells/Colony Forming Assay (North-Root et. al., 1982).**
   e. **Balb/c 3T3 cells/Total Protein (Shopsis and Eng, 1985).**
   f. **BCL-D1 Cells/Total Protein (Balls and Horner, 1985).**
   g. **Primary Rabbit Corneal Cells/Colony Forming Assay (Watanabe et. al., 1988).**

2. **Membrane Integrity**

   a. **LS Cells/Dual Dye Staining (Schaife, 1982).**
   b. **Thymocytes/Dual Fluorescent Dye Staining (Aeschbacher, et. al., 1986).**
   c. **LS Cells/Dual Dye Staining (Kemp et.al., 1983).**
   d. **RCE-SIRC-P815-YAC-1/Cr Release (Shadduck et. al., 1985).**
   e. **L929 Cells/Cell Viability (Simons, 1981).**
   f. **Bovine Red Blood Cell/Hemolysis (Shadduck et. al., 1987).**
   g. **Mouse L929 fibroblasts-Erythrocin C Staining (Frazier, 1988).**
   h. **Rabbit corneal epithelial and endothelial cells/membrane leakage (Meyer and McCulley, 1988).**

i. Agarose diffusion (Barnard, 1989).
j. Living dermal equivalent (LDE). (Bell et. al., 1988).

3. Cell Metabolism

a. Rabbit Corneal Cell Cultures/Plasminogen Activator (Chan, 1985).
b. LS Cells/ATP Assay (Kemp et. al., 1985).
c. Balb/c 3T3 Cells/Neutral Red Uptake (Borenfreund and Puerner, 1984).
d. Balb/c 3T3 Cells/Uridine Uptake Inhibition Assay (Shopsis and Sathe, 1984).
e. HeLa Cells/Metabolic Inhibition Test (MIT-24) (Selling and Ekwall, 1985).
f. MDCK Cells/dye diffusion (Tchao, 1988).

III. CELL AND TISSUE PHYSIOLOGY

1. Epidermal Slice/Electrical Conductivity (Oliver and Pemberton, 1985).
2. Rabbit Ileum/Contraction Inhibition (Muir et. al., 1983).
3. Bovine Cornea/Corneal Opacity (Muir, 1984).
4. Proposed Mouse Eye/Permeability Test (Maurice and Singh, 1986).

IV. INFLAMMATION/IMMUNITY

1. Chorioallantoic Membrane (CAM)

a. CAM (Leighton et. al., 1983).
b. HET-CAM (Luepke, 1985).
c. CAM VA.

2. Bovine Corneal Cup Model/Leukocyte Chemotactic Factors (Elgebaly et. al., 1985).
3. Rat Peritoneal Cells/Histamine Release (Jacaruso et. al., 1979).
4. Rat Peritoneal Mast Cells.Serotonin Release (Chasin et. al., 1979).
5. Rat Vaginal Explant/Prostaglandin Release (Dublin et. al., 1985).
6. Bovine Eye Cup/Histamine (Hm) and Leukotriene C4 (LtC4) Release (Benassi et. al., 1986).

V. RECOVERY/REPAIR

1. **Rabbit Corneal Epithelial cells-wound Healing (Jumblatt and Neufeld, 1985).**

VI. OTHER

1. **EYETEX Assay (Gordon and Bergmen, 1986; Soto et. al., 1988).**
2. **TOPKAT Computer Based/Structure Activity (SAR) (Enslein, 1984, Enslein et. al., 1988).**
3. **Tetrahymena/Motility (Silverman, 1983).**
4. **Microtox**

The potential advantages and disadvantages of in vitro test systems are summarized as follows:

Advantages

1. Avoid complications and potential confounding or "masking" of findings of in vivo studies.
2. Exposure levels and conditions at target sites can be better controlled.
3. Test condition standardization can be better than for in vivo studies.
4. Reduction in animal usage and/or in pain to experimental animals.
5. Ability to directly study some target tissue effects on a real time basis.
6. Reduced requirements for test agents.

Disadvantages

1. Lack of ability to evaluate longer term effects.
2. Limited ability to simulate and evaluate integrated organismic level effects.
3. May not reflect influence of agent absorption, distribution, metabolism and excretion effects.
4. May not detect unique systemic effects resulting from exposure by this route.

What I am referring to in number 4 in the disadvantage test are those cases where animals die very quickly after being dosed in an eye irritation test. Twice during my career, I have dosed one rabbit and have it die before I could dose the second. Others have seen the same (Mass et. al., 1991). If ocular exposure is likely, and a significant volume of the material is to enter use of commerce, at least one animal should receive exposure by this route.

All of the in vitro methods that are available show promise of at least some uses. They have surplanted animal testing to some degree, but not nearly as extensive as might be the case. The first hurdle has been to have them "validated." Though individual investigators have "calibrated" the performance of their own methods using standards of known irritancy (generally of known irritancy in rabbits, while man remains the species of concern), this has not filled the need for scientific test validation. Rather, large intralaboratory tests of multiple methods in multiple

laboratories are required. At least seven such large trials, as summarized in **Table 2** below, have been completed.

TABLE 2:

MULTILABORATORY EVALUATIONS			
ORGANIZATION	**# METHODS**	**# CHEMICALS**	**COMPARATOR IN VIVO DATA**
CFTA (US)	23	20	RABBIT
EEC (EUROPEAN)	7	21	RABBIT
FRAME (UK)	6	56	RAT
MEIC (SW/EUROPEAN)	87	10-50	MAN
PMA (US)	7	35	RABBIT
SDA: PHASES I & II	14	23	RABBIT
ZEBET (GE)	2	35	RABBIT

The results of the multilaboratory evaluations to date can be summarized as follows:

* Ellipsoid effect: Predictive ability for negative and severe irritants/corrosives is generally good, but moderates show poor correlation, with in vivo data.

* To date, batterys do not seem to show improved performance over "best" single tests.

* Single test performance is significantly influenced by structural/functional class of chemical.

* Success of validation programs will depend on expectations or acceptance criteria.

One must also remember that the conduct of in vivo eye irritation tests has also been significantly modified by alternatives, such as (Gad and Chengelis, 1988):

- prescreening for extreme irritants and corrosives by p.H. determinations and dermal irritancy tests.

- use of the less overly sensitive and less stressful low volume test.

- use of fewer animals.

Finally, one must remember that despite their limitations, the rabbit eye tests have successfully identified sever ocular irritants that there is no evidence of such a product entering the market. The often overlooked fourth **R** (after reduction, replacement and refinement) is operative here. It is the **Responsibility** of the industrial toxicologist to prevent unsafe products from entering the marketplace.

In summary, significant progress has been made in developing and evaluating in vitro tests for eye irritation. In my opinion, several of these tests could now serve successfully in place of whole animal tests in screening out those industrial compounds which are not intended to have any eye irritation yet still may present an unacceptable hazard because of their severe irritation potential. However, no single in vitro test (or combination of tests) exists that can currently or in the near future replace all whole animal testing.

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THE ROLE OF *IN VITRO* TESTS IN ASSESSING THE SAFETY OF COSMETICS AND CONSUMER PRODUCTS

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Many cosmetics companies have eliminated animal testing through the use of historical safety data, raw materials supplier's information, and *in vitro* testing.The application of *in vitro* testing to the product development process offers companies the opportunity to improve product quality in a timely, cost efficient manner.

Over the last twelve years, the cosmetics industry in the United States has become one primary target of the animal rights movement. Beauty care companies are particularly susceptible to these attacks because these products are often viewed as non-essential.[1]

The success of the animal rights movement is attributable to its persistence in changing public opinion about the need for safety testing in animals. Its strategy has consisted of public awareness programs, company boycotts, and seeking sponsorship at the state legislative level for bills to limit the use of Draize eye and skin tests. Several multinational cosmetics firms have, as a result, declared moratoriums or bans on animal testing.

The size of the beauty care market is currently estimated at $45 billion dollars.[2] The development of an alternate strategy to eliminate the need for animal tests is essential for cosmetic companies to remain competitive. The purpose of this article is to discuss alternate approaches for assessing the safety of cosmetics and how *in vitro* tests can help improve product quality.

What Is *In Vitro* Testing? What Can It Do?

In vitro testing is testing "in glass," or rather, in a test tube, as opposed to *in vivo*, in a living animal. While *in vitro* testing incorporates the use of living systems, it does not use whole, living, sentient animals. Cell culture lines may be used, as well as single-cell organisms such as bacteria and protozoa to aid in the assessment of a product's safety.

It should be noted that many *in vitro* tests using cell cultures still depend upon animals for a source of cells and tissue. It is important to keep in mind that the sacrifice of one animal (if any sacrifice is needed at all) provides an enormous quantity of cells for *in vitro* testing, thereby greatly reducing the number of animals that would have been used for *in vivo* testing.

In today's product safety testing programs, *in vitro* data is often compared to *in vivo* data in order to assess the *in vitro* test's performance. The ideal *in vitro* test would correlate perfectly with *in vivo* data; in the same token, the ideal *in vivo* test would perfectly predict a product's effect in humans upon accidental or intentional exposure to eye and skin. This is not always the case. No existing *in vitro* or *in vivo* test can predict a product's safety 100% of the time. Still, *in vitro* tests have been found to be reliable and sensitive testing tools. While there is no complete substitute for human or animal data, *in vitro* technology can be effectively implemented by many companies for screening and evaluating products. In addition to reducing and replacing animal testing, there are the issues of time and money that strongly support the notion of *in vitro* testing.

Safety Testing Programs for Consumer Products

Most cosmetic companies have product development schedules in which safety testing programs are conducted at an accelerated rate. These aggressive schedules are maintained to support the seasonal sale periods. It is not uncommon for safety programs for cosmetics to be completed in a 4-6 month period on multiple versions of the same product in order to protect the marketing launch date. The total development time is usually less than a year.

Some cosmetic companies execute product safety testing programs using a tiered approach consisting of pre-clinical testing (Phase 1), clinical testing (Phase 2) and usage studies (Phase 3). This tiered approach has the advantage of providing early safety information on products before large capital investments in equipment and packaging are made. It also provides valuable information about the safety of a product before conducting product usage studies. Marketing studies range from controlled usage tests within the company to national test markets. As a rule, the larger the market the greater the need for completed safety tests and exposure data before clearing a product. It would be unethical to give experimental cosmetic formulations to consumers in a home marketing study without having knowledge of whether the formulation could be an oral toxin or an eye/skin irritant.

Most pre-clinical testing programs are designed to evaluate acute effects such as oral toxicity, ocular and skin irritation and allergic contact dermatitis potential of prototype formulations and product ingredients prior to human panel testing. In a few instances, a new ingredient may require subchronic and chronic testing.

Pre-clinical Testing (Phase 1)

Pre-clinical testing involves conducting animal tests in compliance with Good Laboratory Practice regulations. Usually a minimum of two prototype formulations of each product to reach the marketplace are examined in pre-clinical testing.

Safety departments usually allocate 30-45 working days of the product development schedule to complete the in-life phase of these tests before recommending which prototype formulations should proceed to clinical testing. Approximately 10-20% of the total safety budget for the product may be spent on pre-clinical tests. The characteristics of pre-clinical testing can be found in Table 1.

TABLE 1

Pre-clinical Testing- Phase 1

30-45 working days
Multiple formulations
Good laboratory practice regulations
Cost about 10-20% of total budget
Oral toxicity
Ocular irritation
Comedogenicity
Skin irritation
Allergic contact dermatitis
Phototoxicity and photoallergenicity

Clinical Testing (Phase 2)

Clinical testing involves conducting safety tests on humans under the direction of doctoral level scientists and board certified physicians. Good Clinical Practice regulations are prescribed for these studies in order to protect the rights and safety of subjects involved in such investigations and to help assure the quality and integrity of the data generated from these studies. The data provided from pre-clinical testing serve as a building block for justification of human testing. Occasionally, pre-clinical and clinical programs may be conducted in overlapping time periods. These situations are justified when the composition of similar formulations has already been assessed in pre-clinical testing programs.

Clinical testing programs usually last 60-80 working days and assess multiple prototypes of similar formulations. These tests evaluate skin irritation, allergenicity, comedogenicity and acnegenicity. Typically 30-40% of the safety budget for a product is spent in clinical testing (Table 2). Approval for national test marketing often occurs after completion of the clinical program.

TABLE 2

Clinical Testing- Phase 2

60-80 working days
Multiple formulations
Good clinical practice regulations
Cost about 30-40% of total budget

Cumulative skin irritation
Human allergenicity
Comedogenicity
Phototoxicity and photoallergenicity

Claims Substantiation (Phase 3)

Successful marketing of a cosmetic product often depends on the claims that can be made about a product. Popular claims often associated with cosmetics and skin care formulations include "safe for contact lense wearers", "safe for sensitive skin" and "moisturizes dry skin". Many of these claims are often substantiated with clinical studies conducted under board certified physicians (Table 3). Successful completion of the three testing phases provides justification for national release of a product.

TABLE 3

Claims Substantiation- Phase 3

30-60 working days
Single formulation
Physician as investigator
Good clinical practice regulations
Cost about 60-70% of total budget

Usage studies
Periocular application tests
Acnegenicity studies
Special user group tests

Changes in Safety Testing

While some consumers are delighted with the availability of cruelty free products, others have raised the question of whether or not these products are actually safe. The answers depend upon the philosophy of the manufacturer. Although consumers (including those supporting the animal rights movement) want safe products, there is no consensus among interested parties on how to achieve this goal.

Some manufacturers produce and sell cosmetics with little or no safety testing. They assume that the product is safe because the ingredients are natural as opposed to synthetically manufactured chemicals. This could not be further from the truth. Natural ingredients contain complex molecules that can induce allergic, phototoxic, and/or photoallergic responses. The size of a company has little relationship to whether or not it conducts safety testing.

Some companies that have declared bans or moratoriums on animal testing have developed alternate strategies that allow for the replacement of animal testing using other sources of information. This may entail analysis of structure-activity relationships (SAR) of the ingredients, historical safety data, suppliers' information, and clinical data. Many products can be safely released into the market using this approach (Table 4).

TABLE 4

Modified Pre-clinical Testing- Phase 1

15-30 working days
Multiple formulations
Good laboratory practice regulations
Cost about 5-10% of total budget

SAR
Supplier's safety data
Historical safety records
Small panel usage tests (10-50 people)
Some *in vitro* testing

Not all companies can follow this approach. Pharmaceuticals, medical devices, and insecticides are examples of product types that must comply with specific test requirements for approval from a regulatory agency. Animal testing is usually the key component of the testing requirements.

In Vitro Alternatives for the Draize Eye Test

In vitro tests have been classified into six groups based on endpoint measurement.[3] These endpoints include morphology, cellular toxicity, cell and tissue physiology, inflammation/immunity, recovery/repair and other. The classification of other has been replaced with the term phenomenological. This refers to *in vitro* tests that are based on endpoints that are not biologically relevant to ocular irritation yet have shown promise (through statistically based correlative testing programs) as being useful. Examples of these tests include the *Tetrahymena thermophila* Motility Assay and the Luminescent Bacteria Test. Both tests, which are based on changes in primitive organisms, have shown promise as ocular screening assays for surfactant containing products and raw materials.

The selection of an *in vitro* test battery for predicting ocular irritation should be based on mechanistically relevant changes to the anterior chamber of the eye. Examples of important endpoints include alteration in protein structure (irreversible corneal damage), cytotoxicity, inflammation (e.g. prostaglandins and vasoactive amines) and recovery. A test battery should stress multiple endpoints not necessarily multiple tests. A single test that can measure multiple endpoints will be time efficient and more cost effective than the use of several tests that measure a single endpoint.

A popular test design for assessing the ocular irritation potential of test materials in tissue culture and reconstituted human skin models is varying the concentration while keeping the exposure time fixed. Results are expressed in terms of the effective concentration affecting 50% of the cells (EC_{50} value).

Another test design which more closely mimics the Draize eye test is measuring cellular change at a fixed exposure time and concentration of a test material. The concentration and volume selected for this protocol should be similar to anticipated accidental exposure conditions. This type of design often reduces or eliminates false positive reactions.

The corneal wound closure model offers a mechanistically based test to predict recovery.[4] This test is based on the ability of cultured cells to migrate and recover the standard wound that was inflicted on the model.

The adaptation of LAPS (Light Addressable Potentiometric Sensor) technology to a flow chamber offers an exciting advancement in studying intercellular recovery after pulse exposure to a test material. Good correlations have been reported between *in vivo* ocular irritation tests and *in vitro* tests using the microphysiometer.[5] It is premature, however, to assume that changes in acidification rate can accurately predict ocular recovery. This skepticism is based on the fact the reversible recovery of the cornea involves the movement of water over hours to days into and out of the the stromal layer. The presence of water is the result of an inflammatory reaction mediated by immune cells and vasoactive cells. A relationship must first be established between the acidification rate and inflammation before this can be viewed as a relevant, mechanistically based endpoint.

Managing *In Vitro* Testing

For some toxicologists, the decision to use *in vitro* tests during the developmental cycle of a product would be unthinkable. The reasons most frequently quoted include: "lack of regulatory acceptance", "the technology is too expensive" and *"in vitro tests cannot replace animal testing"*. For these scientists, the use of *in vitro* tests is viewed as experimental and probably presents a risk rather than benefit. Historically, this response is to be expected with the application of any new idea or technology.

The first step in managing *in vitro* testing is to set realistic goals for their application and use. At this time, *in vitro* tests do not possess the level of sophistication and biological integration to be used as stand alone, replacement technology. *In vitro* tests, however, when applied in a cost effective screening program, can provide valuable information about prototype formulations and strengthen the decision to approve the release of a product.

The three logical places to use *in vitro* testing are: at the pre-clinical testing phase (Phase 1), at the claims substantiation phase (Phase 2) and for quality control checks during product manufacturing (Phase 3). During the pre-clinical testing phase, *in vitro* testing results as well as a formulation review may serve as justification for further evolution and controlled in-house panel testing of prototype formulations.

The application of *in vitro* tests to claims substantiation programs can be useful in screening formulations for acceptance prior to clinical testing on sensitive skin individuals. Standard patch design techniques alone are inadequate for judging the mildness of cosmetics, especially for sensitive skin individuals and panelists with pre-existing dermatoses such as seborrheic dermatitis, acne, rosacea and atopic diathesis.

A research project was recently undertaken to examine some of the mediators released from injured reconstituted human skin models (Organogenesis, Inc.).[6] These skin models contain various cells types that could initiate an inflammatory response to cosmetics. The use of *in vitro* skin models eliminates the rapid leakage of these mediators that occurs *in vivo*.[7] The mediators examined include histamine, serotonin, prostaglandin E_2 (PGE_2) and interleukin-1 α (IL-1 α). The properties of these mediators are listed in Table 5.

TABLE 5

Inflammatory Mediators

Histamine is a vasoactive amine released from mast cells as a result of physical and/or chemical induced injury. The *in vivo* effects of histamine are erythema and edema as well as increased capillary permeability and a partial interruption of vascular endothelium.

Serotonin (5-hydroxytryptamine) exists in a preformed state in mast cells. This vasoactive amine causes capillary dilatation, increased permeability and smooth muscle contraction.

Prostaglandin E_2, an inflammatory eicosanoid, is released when the plasma membranes of cells are perturbed. PGE_2 causes increased permeability, dilatation of capillaries and alterations in pain threshold.

Interleukin-1 α is a cytokine produced by keratinocytes in response to injury. IL-1 α is a 15 kD glycoprotein that plays a central role in the initiation and development of an immune response to antigen through its direct effects on the activation, growth and differentiation of T and B lymphocytes.

Two different skin cleanser formulations labelled experimental cleansers A and B were evaluated in this research. The cleansers varied in raw materials and surfactant types and levels (Table 6). Sodium lauryl sulfate and tissue culture media were included in the *in vitro* tests as positive and negative controls, respectively. The results of clinical assessment of these two cleansers can be found in Table 7.

TABLE 6

Cleanser Compositions

Cleanser A	Cleanser B
Water	Mineral oil
Anionic surfactants	Amphoteric surfactants
Amphoteric surfactants	Emollients
Emollients	Botanical extracts
Preservatives	Preservatives
Botanical extracts	Color
Color	

TABLE 7

Clinical Trials

Clinical Trials	Cleanser A	Cleanser B	SLS
Cumulative Irritation (n=20)	moderate irritation	less than mild irritation	moderate irritation
Allergic Contact Dermatitis (ACD) (n=150)	no ACD	no ACD	no ACD
Comedogenicity (n=25)	none	none	none
Acnegenicity (n=150)	positive	negative	not tested
Usage Study Subjective Symptoms (n=150)	stinging, burning, itching	mild itching	not tested

Results from this research showed that the irritation properties of these cleansers were reflected in differences in cytotoxicity and the quantity of histamine (mast cells), serotonin (mast cells), and PGE_2 (fibroblasts and keratinocytes). IL-1 α did not appear to be a discriminating marker for skin irritation. Studies from other laboratories have shown that IL-1 α is more frequently associated with allergic contact dermatitis rather than irritant contact dermatitis.[8] This research shows that the use of selected biological markers can be used to screen surfactant containing formulations prior to conducting more costly clinical testing on panelists with sensitive skin.

The application of an *in vitro* test for biological reactivity is a new concept. The criteria for the release of a cosmetic product are usually based upon visual inspection, pH, viscosity, odor and other physical measurements. An *in vitro* test, when included into a QA release criteria, can detect unexpected changes in the safety of the product, thus strengthening the quality assurance process. Further *in vivo* testing may still be necessary, however.

CONCLUSIONS

The application of *in vitro* testing to the product development process offers companies the opportunity to improve product quality in a timely, cost efficient manner. Too often we think of *in vitro* tests simply as replacements for animal testing. This has limited the acceptance of the technology. Most scientists agree that *in vitro* tests alone can not replace the Draize eye test.

Many cosmetics companies have eliminated animal testing through the use of historical safety data, raw materials supplier's information, and *in vitro* testing. Acceptance of *in vitro* testing will not only require validation, but also educational programs designed to show corporate toxicologists how to use the data for assessing the safety of products.

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ADVANTAGES AND LIMITATIONS OF *IN VITRO* OCULAR TESTING

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The Agar Overlay Assay was more predictive of eye irritation than the MEM Elution Assay. The CAM assay correlated well with Agar Overlay in predicting conjunctivitis and iritis but did not predict corneal anesthesia, discharge, onset, duration, corneal opacity or rinse mitigation. The cell culture and chick embryo assays with pharmaceuticals and pesticides were good predictions of reversible conjunctivitis. Data will be presented with the Draize test through use of a complex series of assays (vascular, secretive, autonomic, coagulation) where data generation cannot merely be thought of as a number or score.

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APPLICATIONS OF *IN VITRO* TOXICOLOGY TO CORPORATE AND REGULATORY OCULAR SAFETY DECISIONS

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Product safety is ultimately a corporate responsibility. While government regulatory oversight contributes significantly to the process of product safety evaluation in many industries, many safety decisions are made at the corporate level. Efforts to reduce or eliminate animal-based safety testing and the desire for more quantitative data, have increased the focus and reliance on *in vitro* assays. Considerable debate has been raised over the appropriate methods for evaluating the efficacy of *in vitro* methods as predictors of *in vivo* ocular irritancy. Successful approaches have been divided into four phases. First, determination of what is expected of the *in vitro* test. For example, is classification into broad categories of irritancy acceptable. Second, identification of a reference set of materials which represent the desired class of chemicals and for which sound *in vivo* data are available. Third, design and execution of a scientifically sound evaluation of the candidate *in vitro* assays. Fourth, evaluation of performance of each test in the battery based on the original requirements. Such programs have allowed *in vitro* assays to become part of an overall tiered safety testing program.

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Dr. John W. Harbell, Program Manager, Division of *In Vitro* Toxicology, Microbiological Associates, Inc., received his Ph.D. from the University of California. He spent five years with the Army's Clinical Research Program where his research focused on development of tailored chemotherapeutic regimes for the treatment of human tumors. Human tumor cell panels were used to assess drug efficacy and interaction. In 1984, he became the director of the Army's Genetic Toxicology Laboratories. He joined Microbiological Associates, Inc. in 1987 where he developed and used *in vitro* model systems for the study of mammalian cell mutagenesis, ocular irritation and skin irritation. He is the co-chair of the Cellular Toxicology Committee on the Tissue Culture Association.

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Session IV: *In vitro* and Other Alternatives in Inhalation Toxicology

Co-Chairs: Drs. Sandra Thomson and Richard D. Thomas

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Sandra Thomson is currently employed by the U.S. Army Chemical Research, Development and Engineering Center (CRDEC), Research Directorate, Toxicology Division, Aberdeen Proving Ground, MD as the Chief of the Inhalation Toxicology Branch. Previously (1979) she worked as a toxicologist for the U.S. Army Environmental Hygiene Agency. Prior to Federal Civil Service, she taught at Harford Community College. She is a Phi Beta Kappa graduate of the University of Connecticut (B.A., Zoology, Chemistry, 1966, Ph.D., Biochemical Nutrition, 1970).

At CRDEC, Dr. Thomson is the senior researcher and team leader for inhalation studies on chemicals of military interest and has served as an Acting Chief of Environmental Toxicology Branch in Toxicology Division. She has numerous publications in the field of Smoke/Obscurant Toxicology and on the biochemical and cytological effects of airborne chemicals on the mammalian pulmonary system. In 1987 Dr. Thomson received the prestigious U.S. Army Research and Development Achievement Award for Research on the effects of inhaled particulates on pulmonary alveolar development by the U.S. Army Ballistics Research Laboratory for dust concentration monitoring in their safety program for test ranges. As a member and consultant for several CRDEC Weapons Systems Management Teams, she has been a liaison with academic and industrial representatives of the Smoke/Obscurant Community.

In addition to membership in the Association of Government Toxicologists, Dr. Sandra Thomson belongs to the American Conference of Governmental Industrial Hygienists (ACGIH), American Industrial Hygiene Association, National Capital Area Chapter of Society of Toxicology, and the Society of Toxicology.

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In Vitro and Other Alternatives in Inhalation Toxicology

Monitoring Biologic Markers of Cellular and Biochemical Response

Richard D. Thomas

In recent years, much has been learned concerning the cellular and biochemical mechanisms of lung response to both chemical insult and disease. This presentation examines the rapidly developing field of cellular interactions and biochemical mechanisms of respiratory response. Its focus is the work we have completed at the National Research Council/National Academy of Sciences on "Biologic Markers in Pulmonary Toxicology" (NRC, 1989a). Other markers reports in this series may also be of interest (NRC, 1989b, 1992a,b).

There are several possible sources of biologic markers that may be monitored in *in vitro* systems to understand lung and respiratory tract toxicity. These techniques are relatively new and still entail some problems in their application for studying biologic models. Many of these new techniques and model systems will be described in this session.

The following is an overview of the cellular and the acellular components of the respiratory system that may be monitored for biologic markers of toxic response.

MACROPHAGES

The predominant cell in BAL fluid from human and animal subjects is the macrophage. In some species (Henderson, 1988a), lymphocytes can be present in small numbers. Neutrophils, eosinophils, and mast cells might also be present as a result of an inflammatory response.

Human alveolar macrophages (AMs) are composed of several populations that can be distinguished by density. In general, denser AMs are less mature and resemble blood monocytes more than less dense AMs. The denser cells are more potent producers of a soluble factor that inhibits fibroblast proliferation (Elias et al., 1985a) and of interleukin-1 (Il-1) (Elias et al., 1985b), and they are more efficient accessory cells for antigen-induced proliferation (Ferro et al., 1987). AM abnormalities in sarcoidosis might represent differences in the relative proportions of AM subpopulations, rather than intrinsic differences in the same AM subpopulations. Hance and colleagues (1985) have found that AMs from patients with sarcoidosis express antigens that are present on blood monocytes, but AMs from normal subjects do not. Other attributes of sarcoid AMs, such as accessory cell function (Venet et al., 1985) and spontaneous release of interferon-gamma and growth factors (Bitterman et al., 1983), are compatible with the influx of less mature AMs in sarcoidosis.

Macrophages are important in the regulation of the immune response, acting as both promoters and suppressors of events that result in immunization or inflammation (Unanue et al., 1984). In general, AMs that can be obtained from normal humans with lavage contain

subpopulations that can increase or suppress lymphocyte proliferation. The result depends on culture conditions—the presence of other accessory cells and the amount of antigen or mitogen present (Liu et al., 1984; Toews et al., 1984; Ettensohn et al., 1986). Alterations in accessory cell function might affect pathogenesis. In fact, as mentioned above, some studies have shown that AMs from patients with sarcoidosis have accessory cell function more efficient than that in AMs from normal subjects (Lem et al., 1985; Venet et al., 1985). AMs from asthmatics are less able than AMs from normal subjects to suppress mitogen-induced lymphocyte proliferation (Aubas et al., 1984). Changes in accessory cell function are gaining acceptance and should be used as markers of environmental exposure and perhaps as markers of susceptibility to disease.

Interleukin-1

AMs can secrete Il-1, but apparently to a smaller degree than can peripheral blood monocytes (Koretzky et al., 1983; Wewers et al., 1984). The uncertainty regarding AM Il-1 production and its control mechanisms probably derives from the multiplicity of AM products, some of which antagonize Il-1 production (Monick et al., 1987). Il-1 is an important mediator of inflammation (Dinarello, 1984) and acts as a differentiation signal for several subsets of lymphocytes (NRC, 1992a). In particular, Il-1 promotes differentiation and maturation of helper T cells (Mizel, 1982), lymphocytes (Rao et al., 1983), and natural killer cells (Dempsey et al., 1982). Il-1 is chemotactic for neutrophils, can alter the adherence properties of endothelial cells by stimulating prostaglandin synthesis (Bevilacqua et al., 1985), and can induce fibroblast proliferation (Schmidt et al., 1982). In animals, perturbation of the lung can cause spontaneous release of Il-1 (Lamontagne et al., 1985). An analogous situation can occur in sarcoidosis, in that Il-1 is spontaneously released *in vitro* in the absence of additional signals (Hunninghake, 1984). AM Il-1 secretion is influenced by the cytokines, such as interferon-gamma. AMs from normal subjects and persons with lung disease might differ in their response to stimuli that increase Il-1 secretion (Eden and Turino, 1986).

Interferon

AMs from normal subjects can release interferon (-alpha or -gamma) after stimulation by appropriate inducers. It is interesting that cigarette-smoking does not influence inducer-stimulated interferon secretion (Nugent et al., 1985). However, AMs from patients with sarcoidosis spontaneously release interferon without the necessity of inducers (Robinson et al., 1985).

Reactive Oxygen Species

AMs release reactive oxygen intermediates, such as O_2^-, H_2O_2, OH^-, and singlet oxygen (NRC, 1992a). Those products have many proinflammatory effects, including inactivation of protective substances in the lung and cytotoxicity. Changes in the signals required for release of those products or changes in the signals controlling the release of protective substances, such as superoxide dismutase, could have important implications regarding the results of exposure to injurious stimuli. For example, the production of oxygen radicals is increased in AMs from smokers (Hoidal et al., 1981) and from subjects with sarcoidosis (Greening and Lowrie, 1983). Interferon-gamma can increase *in vitro* oxygen radical production (Fels and Cohn, 1986). AMs from asthmatics

differ from those from normal subjects, in that they have increased numbers of IgE receptors and require only exposure to antigen to secrete reactive oxygen products, whereas AMs from normal persons must first be coated with IgE antibody (Joseph et al., 1983). Release of other AM products, such as platelet activating factor and neutrophil and eosinophil chemotactic factors (possibly leukotriene B_4), exhibit the same dependence on surface IgE antibody (Gosmset et al., 1984). AMs from normal animals do not release reactive oxygen intermediates after stimulation with phorbol esters, unless they are conditioned *in vitro* with serum; AMs from animals with pulmonary inflammation do not require conditioning to become responsive to phorbol esters (Gerberick et al., 1986). AM sensitivity to stimuli that cause secretion of toxic oxygen intermediates might be a useful marker of lung damage and inflammation.

Chemotactic Factors

AMs release chemotactic factors for neutrophils (Hunninghake et al., 1980a), whose products are important in the pathogenesis of several types of lung injury, such as adult respiratory distress syndrome (Lee et al., 1981; Tate and Repine, 1983) and bronchopulmonary dysplasia. AMs also release factors that stimulate neutrophil adhesiveness and superoxide generation (Tate and Repine, 1983). Cigarette smokers' AMs, unlike nonsmokers' AMs, spontaneously release a neutrophil chemotactic factor *in vitro* (Hunninghake and Crystal, 1983). Other environmental exposures might similarly change the signals needed to cause release of chemotactic factors.

Miscellaneous Factors

Pulmonary macrophages can release a factor that causes the release of histamine from human basophils and lung mast cells (Schulman et al., 1985). Alterations in the mechanisms that control the release could both cause inflammation and be a result of environmental exposure. AMs release growth factors that influence diverse cell types, such as fibroblasts (Chapman et al., 1984), smooth muscle cells (Martin et al., 1981), endothelial cells (Martin et al., 1981), and Type II cells. Although Il-1 is one of those factors, it is clear that additional substances are released by macrophages (Leslie et al., 1985). The proliferation of many lung cell types that is a common result of inflammatory stimuli could be caused by AM growth factors, and release of the factors could be an early event in lung changes and provide markers of early lung injury.

NEUTROPHILS

Neutrophils are not usually found in large numbers in BAL fluid from normal lungs (Reynolds, 1987; Henderson, 1988a). In lavage fluid from the lungs of smokers (Young and Reynolds, 1984) or from subjects that were exposed to ozone (Seltzer et al., 1986; Koren et al., 1989), an increased percentage and greatly increased absolute numbers of neutrophils are found. As participants in the inflammatory response in lung tissue, neutrophils migrate into the lung from the blood under the influence of one of several chemotactic factors, including complement component C5a and soluble factors secreted by AMs. They might be considered as a secondary line of phagocytic defense for the lungs, which can be recruited into the airspaces in response to exposure to microbial agents or other inhaled materials.

The neutrophil itself contains a host of materials potentially damaging to lung tissue (Henson, 1972; Martin et al., 1981). It has the capacity to release oxygen radicals. In addition, it releases several digestive enzymes, especially neutrophil elastase. The release of neutrophil elastase has been associated with significant lung destruction (Janoff, 1972), as seen in the adult respiratory distress syndrome, where it has been found in lavage fluid (Lee et al., 1981). Neutrophil elastase can cause emphysematous changes in an animal model and has been proposed as a major cause of emphysema. Other less destructive processes have been associated with the release of enzymes from the neutrophil.

LYMPHOCYTES

The lymphocytes in lung fluid usually are T cells, although natural killer cells and B cells have also been found. The importance of B cells and NK cells in the lung is unclear, inasmuch as these cells are susceptible to the suppressive effects of surfactant and other substances in the lung lining fluid. T cells have been divided into two classes on the basis of the monoclonal antibodies directed against antigens found on the surface of the T cells. For example, CD4 cells are in the Th/i cell class, and CD8 cells are in the TS/c cell class.

Regardless of the cell type responsible for antigen presentation in the lung, antigen must be presented to stimulate T-cell proliferation. It is not clear whether antigen is presented in the lung or in the lymph nodes that drain the lung. Results of investigations in dogs and nonhuman primates suggest that antigen deposited in the lung is translocated to draining lymph nodes (Kaltreider et al., 1977), where proliferation of antigen-specific B cells occurs (Bice et al., 1980b). The antigen-specific B cells then enter the blood and are recruited by nonspecific means to the lungs (Bice et al., 1982). With respect to secondary antigen challenges, results of studies in cynomolgus monkeys suggest that local proliferation of lymphocytes can occur in the lung (Mason et al., 1985). Little is known of primary immune responses in the human lung, but mechanisms responsible for the accumulation of T cells in the lungs of patients with sarcoidosis have been the subject of numerous investigations.

It has recently been shown that T cells in the lower respiratory tract of patients with active sarcoidosis proliferate spontaneously (Pinkston et al., 1983) and release Il-2 (Hunninghake et al., 1983). The major sources of Il-2 are T cells that express human leukocyte antigens-D region (HLA-DR) on their surface (Saltini et al., 1986). Il-2 secretion is probably associated with the pulmonary lymphocyte infiltrate in sarcoidosis. When II-2 release by lung T cells is suppressed by treatment of patients with corticosteroids, the spontaneous proliferation of T cells ceases, and the numbers of T cells and disease activity subside (Ceuppens et al., 1984; Pinkston et al., 1984; Bauer et al., 1985; Rossi et al., 1985).

A relatively recent investigation determined that Il-2 gene expression by lung T cells was enzymes in response to inhaled toxic particles (Wolff et al., 1988).

PROTEIN AND PROTEIN PRODUCTS

Protein in BAL fluid is measured as a marker of increased permeability of the alveolar-capillary barrier and is a common component of the inflammatory response. Bell and Hook (1979) reported that 80% of the soluble protein in human BAL fluid could be accounted for by 19 plasma proteins. The protein content indicated a preferential transfer of smaller proteins across the

alveolar-capillary barrier. IgG and IgA constituted a higher fraction of total protein in BAL fluid from smokers than in serum (Bell et al., 1981). Transferrin was the only nonimmunoglobulin protein with a higher concentration in lavage fluid than in serum. Serum proteins in BAL fluid from animal studies have proved to be sensitive markers of the inflammatory response (Alpert et al., 1971; Bignon et al., 1975; DeNicola et al., 1981; Beck et al., 1982; Lehnert et al., 1986).

The amino acid hydroxyproline is a marker of collagen and has been interpreted as a marker of collagen breakdown. Hydroxyproline content of BAL fluid has been measured as a marker of breakdown or remodeling of pulmonary collagen in ozone-exposed rats (Pickrell et al., 1987). The increase in hydroxyproline in BAL fluid appeared to parallel developing pulmonary fibrosis in hamsters and rats exposed to diesel exhaust (Heinrich et al., 1986; Henderson et al., 1988).

Exposure to environmental toxicants can cause damage in single cells at the level of DNA, and that damage can lead to the development of many diseases, including cancer. Toxicant-induced changes in specific (although often unidentified) genes are thought to be the initial events in the development of disease. Identification of genes involved in the development of specific diseases can lead to improved diagnosis, understanding, and treatment, but is not essential. In lieu of disease-specific molecular markers that could be used to study the relationship between toxicant exposure and the development of disease, the general interaction between toxicants and DNA can serve as a source of molecular markers of exposure, effect, and susceptibility. The use of molecular markers, defined here as alterations in DNA or RNA, to identify cellular responses or responsiveness to environmental toxicants theoretically can provide information useful in determining the magnitude of exposure, the effects of exposure on human health, and the mechanisms of response.

Molecular markers can be highly sensitive and specific indicators of cell damage or change. Detection of toxicant-induced alterations and use of them as indicators of toxicant exposure, effect, or susceptibility depend on several factors, including the frequency of the alteration, which in turn can affect the sample size required for its detection; the availability of sufficient material (DNA, RNA, or cells) or analysis; and the ability to culture cells from the respiratory tract. The following presentations in this session will focus on the monitoring of these cellular and molecular markers in various cells from the respiratory tract, grown in *in vitro* cultures.

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ALTERNATIVES TO *IN VIVO* TOXICOLOGIC TESTING OF RODENT AIRWAY EPITHELIA

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ABSTRACT

In order to better define the responses of lung cells to potentially pathogenic insults, primary cell cultures of dissociated respiratory epithelial cells have been established. These epithelial cells have been obtained from areas of the respiratory tract ranging from the trachea to the alveolus and the cultures have been demonstrated to mimic the differentiated state of these cell types observed *in situ*. Several procedures which enhance differentiation have been evaluated, which include maintenance on more physiologically-relevant substrata, such as collagen gels, use of defined serum-free medium and use of air/liquid interface systems. These approaches have allowed better definition of cellular responses of respiratory epithelium to toxic insults.

The lungs and airways are susceptible to insult from a wide variety of toxicants. Some toxicants are absorbed through non respiratory routes, but many are delivered directly into the lung or airways. Respirable toxins may be gases, liquid aerosols, or particulates. The first contact of these toxins is with the epithelium of the airways and the overlying surface liquid layers.

In vivo studies of the effects of respirable toxins in humans are generally limited to low dose studies of air pollutants or accidental exposures. *In vivo* studies in animals have proven valuable but there are drawbacks: inhalation studies in animals are expensive, laborious, and it is often difficult to create appropriately controlled exposures. Direct toxin effects on airways can be difficult to distinguish due to inflammatory, immunologic, neuronal and hormonal responses which accompany the insult. We will review some advantages and disadvantages of *in vitro* systems and describe the primary cell culture system used in our laboratory.

In vitro systems lack hormonal, neuronal, inflammatory, immunologic, and other systemic influences. This is both the single most important advantage, and disadvantage, of such systems. It is an advantage in that any observed response of the respiratory tissue is due to that tissue itself, without the confounding influence of other body systems; this makes *in vitro* systems excellent for mechanistic studies. It is a disadvantage in that the response to a particular stimulus in a live animal will be at least partially dependent on these same systemic influences. Ultimately, information gained via an *in vitro* method will be used to understand responses in a live animal. Since the live animal will have the systemic influences which the *in vitro* model lacks, observed responses in the model may be at variance with responses in the live animal. Additionally, such isolated *in vitro* systems can provide no information on how a potential respiratory toxin would affect other organ systems.

Certain chemical compounds exert little effect in their native state but have profound influence after becoming biologically activated. While the lung has the capacity for metabolic activation and contains P-450 monoxygenases, several compounds are activated outside the lung, often in the liver, and then exert pathologic effects on respiratory target tissues. If such a substance were tested in it's native state using an *in vitro* respiratory cell system, a false impression of it's toxic potential might result.

In vitro systems allow increased control of experimental variables. Dosages, exposures, temperatures, and many other factors can be controlled more closely *in vitro* than *in vivo*. Animal to animal variations include not only age, sex, weight, or strain differences, but also many variables which are difficult to assess, such as the presence of subclinical infections or somatic mutations. Many *in vitro* models allow several treatments to be evaluated on tissue from the same source, keeping variations as small as possible.

Tissues *in vitro* may be morphologically or functionally different from the same tissue types *in vivo*. Living animals maintain a remarkable homeostasis; when provided with food, water, and minimal shelter, they closely regulate their own temperature, electrolyte balance, and other vital parameters. *In vitro* systems depend on the investigator entirely for provision of nutrients, oxygen, temperature regulation, defense from pathogens, etc. Unless all conditions can be mimiced exactly as they would be *in vivo* (as of yet a near impossibility), diffrences between tissue *in vitro* and *in vivo* are to be expected. It is always a goal to use tissues as similar as possible to those *in situ*, but it is our responsibility to acknowledge the differences between the tissue *in vitro* and *in situ*, as well as the similarities.

An advantage of *in vitro* systems which cannot be over emphasized is the minimization of animal distress. An animal which is humanely sacrificed to provide tissue undergoes minimal stress compared with an animal subjected to repeated handling or application of inhaled gasses or particulates. Tissues used in *in vitro* methods must at some point come from an animal, but many of these methods allow the use of fewer animals than would otherwise be necessary. For example, cell cultures allow tremendous expansion of tissue, and transformed cell lines provide expandable tissue with a virtually unlimited life span.

The *in vitro* model used in our laboratory involves a primary, biphasic cell culture system. Cell culture techniques can involve primary culture of cells dissociated from the respiratory system or passaged cell lines. Primary culture involves the enzymatic dissociation of cells and their distribution in a nutrient media on a substrate which allows cellular attachment, spreading, and growth. Subcultures of primary cultures, or virally induced immortalization may give rise to permanent cell lines. Immortalized cells may be quite different from the progenitor cells taken from the animal. Even primary cell cultures may be morphologically and functionally disparate from the same cell types *in vivo*. A prime feature of epithelial cell culture is that since respiratory epithelium is not vascularized *in vivo*, the *in vitro* need to derive nutrients and oxygen by diffusion is not abnormal for this tissue.

Cell cultures, like all experimental systems, have advantages and disadvantages. Newer techniques allow cells in culture to more closely resemble the *in situ* differentiated cells. Since a large mass of epithelial tissue is available, several treatments may be applied to cells from one animal, resulting in improved uniformity of results. Cell lines are genetically alike and allow maximum control of experimental variables. Dissociated cells can be enriched for a certain population before culturing. The greatest advantage of cell culture is that only one tissue type (i.e., epithelial cells) is present, insuring that a response is indeed due to epithelial cells. Primary cultures tend to assure a cause and effect relationship. On the down side, cells of primary cultures are exposed to proteolytic enzymes during collection, and the relationship of epithelial cells to other tissues (such as connective tissues) is lost completely. In many culture systems the epithelia loose their differentiated cellular phenotype, which is critically dependent upon the growth medium and on the support surface.

Our cell culture system utilizes guinea pig tracheal epithelium and maintains a remarkably well differentiated, polarized phenotype. After enzymatic dissociation with pronase, cells are resuspended in Dulbecceo's modified Eagles medium/Ham's nutrient mixture F12 and 5% fetal bovine serum and plated in special chambers atop a collagen coated nitrocellulose filter. The cells are fed from above and below with the culture media containing insulin, transferrin, hydrocortisone, epidermal growth factor, cholera toxin, and bovine hypothalamus extract. During the first several days bovine serum is added to the medium, but on about day 4 the serum is no longer used. After these first few days in culture, medium is no longer added above the filter, leaving the top of the cells (the apical surface) exposed to air, as *in vivo*. After 8 days, the cells form a pseudostratified columnar epithelium nearly identical morphologically and functionally to mucosal epithelium *in vivo*.

Morphologically, ciliated, goblet, and basal cells are present in these cultures. The cultures have a virtually identical volume density for all 3 major epithelial cell types as that found in tracheal epithelium *in situ*. The volume density of granule content within secretory cell cytoplasm is almost the same as that *in situ*. The major differences between our cells and guinea pig tracheal epithelium *in situ* are fewer total cells per area, a slightly reduced height to the pseudostratified columnar epithelium, and fewer basal cells per mm basement membrane.

Glycoconjugates released from these cells are mucin-type. The hyaluronidase resistent high molecular weight glycoconjugates have an amino acid composition almost identical to that of mucin, with about 30% serine and threonine residues. There are negligible amounts of

chondroitin or dermatan sulfates. Bouyant density of the glycoconjugates are essentially the same as that of respiratory mucins. Most of the high molecular weight glycoconjugates are secreted apically, suggesting a polarity of secretion as well as of morphology. This system retains the advantages of previous cell culture systems while maintaining normal cell orientation, polarity, and epithelial interactions.

The majority of the work from our laboratory involves the use of these primary cultures to characterize effector type functions of the airway epithelium. Classically, tracheal epithelium has been thought of as a tissue which respond to stimuli in a somewhat passive manner, e.g. by secreting more mucus, altering ciliary beat frequency, or undergoing hypertrophy, hyperplasia, or metaplasia. It has become increasing clear recently that tracheal epithelium can act as an effector; it can act in an autocrine or paracrine manner to alter both it's own fate and the fate of neighboring cells as well. This epithelium produces a number of mediators which either directly or indirectly change the outcome of an encounter with a stimulant. Examples of substances produced by the tracheal epithelium include eicosanoids (prostaglandins, leukotrienes, and HETES), PlateIt activating factor, cytokines, reactive oxygen species, epithelial derived relaxing factors, and extracellular matrix components.

Conclusions

In vitro models of airway epithelium offer both advantages and disadvantages over *in vivo* models. The elimination of systemic influences and the isolation of one tissue type afforded by cell culture provide an important system for the study of mechanistic reactions in the airway epithelium. Our unique, biphasic primary cell culture system using Guinea pig tracheal epithelium has many structural and functional similarities to the same tissue *in situ*. We use this system extensively in the study of the basic biology of these cell types, and the system offers an excellent opportunity for the study of airway toxins as well.

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UNDERSTANDING MECHANISMS OF CARCINOGENESIS USING RAT TRACHEAL EPITHELIAL CELLS IN VITRO

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In vitro transformation assays are used to increase understanding of mechanisms of carcinogenesis and to determine the transforming potency of individual agents. While these assays can provide information on the mechanism for specific steps in the carcinogenic process or can predict the carcinogenic potency of a given toxicant, the relevance of *in vitro* endpoints or responses for carcinogenesis must be determined. This paper will focus on the utility of an *in vitro* system for understanding the mechanisms of multistage respiratory carcinogenesis and on considerations that are critical for its use and application.

To understand the process of malignant transformation of respiratory epithelial cells, it is necessary to identify mechanisms controlling proliferation and differentiation of normal respiratory cells and to define changes in these mechanisms as cells undergo multistep progression towards malignancy. Cell culture systems and studies with isolated cells are potentially useful experimental models for such investigations. Using cell culture systems, cellular and molecular mechanisms involved in carcinogenesis can be investigated under conditions devoid of host-mediated modulating factors. A model system using rat tracheal epithelium as the target tissue has been developed for studies of experimental respiratory carcinogenesis[1]. This model has several useful features. First, the histological structure of the rat trachea resembles that of the human bronchus, a major site of lung cancer in humans[2]. Second, rat tracheas exposed to carcinogens express a range of preneoplastic and neoplastic lesions[3,4]. Third, the development of preneoplastic and neoplastic rat tracheal cell variants can be quantified in cell culture following exposure of tracheal cells to carcinogens either *in vivo* or *in vitro*[5-7]. Fourth, following additional proliferation *in vitro*, preneoplastic variants can progress to neoplastic cells that are capable of forming squamous cell carcinomas when injected into syngeneic or immuno-suppressed hosts[8-10]. Thus, rat tracheal epithelial (RTE) cells representing a range of phenotypes from normal to neoplastic can be isolated and used to characterize both individual stages of progression and the mechanism of progression to neoplasia.

However, *in vitro* model systems used to study mechanisms of carcinogenesis or to identify potential carcinogens from exposures to toxicants must accurately reflect *in vivo* events and risks since the ultimate goal of developing and using an *in vitro* model system is to understand or predict mechanisms or risks of carcinogenesis *in vivo*. This paper describes studies using the RTE cell model for experimental carcinogenesis to address two questions:

1) Are the events observed during the multistep progression of RTE cells to neoplasia *in vitro* the same as those involved in respiratory carcinogenesis *in vivo*?

2) Can the *in vivo* carcinogenic risk for suspected lung carcinogens be estimated from the *in vitro* response of RTE cells to those carcinogens?

Question 1 - Does progression *in vitro* = progression *in vivo*?

There are many similarities between changes seen during the neoplastic progression of respiratory cells *in vitro* and *in vivo*. For example, a change that may be important at an early stage of transformation *in vitro* or of carcinogenesis *in vivo* involves the autocrine production, by altered cells, of transforming growth factor alpha (TGF α). Normal RTE cells in culture require exogenously added epidermal growth for maximal proliferation. In contrast, preneoplastic and neoplastic RTE cells in culture exhibit maximal proliferation without the addition of epidermal growth factor to the culture medium[11,12]. This may be due to production of TGF α by transformed RTE cells resulting in an autocrine stimulation of cell proliferation[12]. These results suggest that the increased expression of TGF α may be a critical, early event in the transformation of RTE cells *in vitro*. Similarly, hyperplastic and neoplastic lesions induced in the lungs of rats that inhaled $^{239}PuO_2$ express increased levels of TGF α; 65% of proliferative lesions and 72% of rat lung tumors were found to express TGF α[13]. Similarly, increases in TGF α expression have been observed in human lung tumors[14] and in preneoplastic and neoplastic lung lesions in dogs[15]. These results suggest that changes in the expression of TGF α are important at an early stage of respiratory carcinogenesis *in vivo*. Thus, understanding the role and mechanisms of increased TGF α expression in RTE cells *in vitro* may provide information useful in understanding the role and the mechanism of development of this change in carcinogenesis *in vivo*.

A change that may be important at a later stage of progression to neoplasia is the acquisition of resistance to the proliferation inhibitory effects of transforming growth factor beta (TGF β). Neoplastic RTE cells *in vitro* and cells derived from dog and many human lung tumors are resistant to the inhibitory effects of (TGF β), whereas their normal and preneoplastic counterparts are much more sensitive[14,16]. As above, understanding the mechanism of resistance to TGF β in cultured RTE cells is likely to improve our understanding of the role that the genetic change(s) identified by this phenotype play during carcinogenesis *in vivo*. These and other similarities between specific changes apparently involved in the neoplastic progression of respiratory cells *in vivo* and *in vitro* suggest that *in vitro* models can be used to study specific changes involved in neoplastic progression and their mechanisms of development.

Differences have also been observed in the specific changes that occur during carcinogenesis or transformation of respiratory epithelial cells *in vivo* and *in vitro*. Although preneoplastic and neoplastic RTE cells *in vitro* produce TGF α, they do not exhibit an increased expression of its receptor, the epidermal growth factor receptor (EGFR)[12]. However, increased expression of the EGFR has been observed in a fraction of preneoplastic and neoplastic lung lesions in rats[15] and dogs[17]. Several possibilities for this difference in the expression of the EGFR in respiratory cells *in vitro* and *in vivo* are discussed below; however, since changes observed *in vivo* are not observed *in vitro*, the *in vitro* RTE cell model of carcinogenesis may not be a useful model for understanding either the role of increased expression of the EGFR in carcinogenesis or the mechanism by which it is over-expressed.

It has been shown that up to 40% of the proliferative and neoplastic lesions in the lungs of rats which inhaled $^{239}PuO_2$ have mutations in the first position of the 12th codon of a Ki-*ras* proto-oncogene[18]. These observations suggested that Ki-*ras* mutations may be important early changes in the development of some lung cancers. To determine if the same changes in Ki-*ras* were induced during the preneoplastic transformation of RTE cells by alpha radiation, we have examined the Ki-*ras* gene of radiation- and chemical-induced variants for point mutations in the 12th, 13th, or 61st codons. In contrast to the *in vivo* results, none of 16 total preneoplastic RTE cell variants examined (9 alpha-particle-induced, 4 X-ray-induced, 3 chemical-induced) had Ki-*ras* mutations. These results suggest that the *in vitro* RTE cell model of carcinogenesis may not be a useful model for understanding either the role of Ki-*ras* mutations in respiratory carcinogenesis or the mechanism by which they develop.

These differing *in vivo* and *in vitro* results suggest at least three possible explanations.

(1) The *in vitro* RTE cell transformation assay may detect earlier events than can be identified histologically during carcinogenesis *in vivo*, and Ki-*ras* mutations and over-expression of the EGFR may not be critical events at this early stage of transformation. If this explanation proves to be true, then the development of these changes may occur at later stages of RTE cell transformation and an understanding of their development *in vitro* could still be useful for characterizing their role and development during carcinogenesis *in vivo*.

(2) Pathways of carcinogenesis for proximal air way cells (*in vitro* results with RTE cells) and peripheral lung cells (*in vivo* results presumably involving Type II cells[19]) are different. If this explanation proves to be true, then results obtained using RTE cells *in vitro* may only be useful in understanding RTE cell carcinogenesis *in vivo*. *In vitro* models using peripheral lung cells may have to be developed to study the development of peripheral lung tumors. Although there are similarities in the types of changes observed for the histologically different types of lung tumors, differences similar to those described here have also been described between histotypes[20,21].

(3) Pathways of carcinogenesis for respiratory epithelial cells are different *in vivo* and *in vitro*. If this explanation is proven to be true, then *in vitro* models of respiratory carcinogenesis will not be very useful in understanding carcinogenesis *in vivo*.

Although we cannot distinguish between these possibilities at the present time, the differing

in vivo and *in vitro* results described above demonstrate that *in vivo* mechanisms of carcinogenesis cannot simply be deduced from an *in vitro* system without careful validation and interpretation of the model being used. In addition, these results demonstrate a need for *in vivo/in vitro* comparisons using model systems that are as closely related as possible.

Question 2 - Does transformation risk *in vitro* = carcinogenic risk *in vivo*?

As noted above, the RTE cell model for experimental carcinogenesis enables cells to be exposed to suspected carcinogens either *in vivo*, before the isolation of RTE cells for analysis in culture, or *in vitro*, after the cells have been isolated and plated in culture[5,6]. We have compared the *in vitro* transformation responses of RTE cells *in vitro* following exposure of cells to alpha particle radiation or ozone either *in vivo* or *in vitro*.

RTE cells were exposed to alpha particles *in vivo* by exposing rats to radon progeny[22]. Following exposure, cells were isolated, placed in culture, and the frequency of radiation-induced preneoplastic variants was quantified. Similarly, RTE cells were exposed to alpha particles *in vitro* by exposing cultures to electroplated sources of ^{239}Pu[23]. RTE cells isolated from animals given increasing exposures to radon progeny and RTE cells exposed to ^{239}Pu in culture exhibited exponential decreases in survival and increases in their frequencies of preneoplastic transformation. These results suggest that RTE cells have similar responses to alpha particles *in vitro* and *in vivo*.

The opposite type of result was obtained when RTE cells were exposed to ozone *in vivo* or *in vitro* and were examined for preneoplastic transformation. RTE cells isolated from rats exposed to ozone (maximum total exposure of 1.2 parts per million of ozone, 6 hours per day, 5 days per week for 4 weeks) had no increase in their frequency of preneoplastic variants compared to air-exposed controls[24]. However, ozone exposures *in vivo* were not without effect since focal attenuation of the epithelium and loss of cilia were observed in the tracheas of animals exposed to ozone. In contrast, RTE cells exposed to ozone *in vitro* (0.7 parts per million ozone, 40 minutes, two-times per week for 4.5 weeks) exhibited 2- to 3-fold increases in their frequency of preneoplastic transformation compared to control cultures exposed to air[21]. Although the exact ozone exposures received by RTE cells in these different experiments is not known, these results suggest that RTE cells exposed to ozone *in vivo* or *in vitro* do not exhibit the same transformation response. Although it is not known if RTE cells *in vivo* are protected from ozone by mucus, if the cells are better able to repair ozone-induced damage, or if they simply are not transformed, these results, whatever the reason for the differences in responsiveness to ozone following exposures *in vivo* or *in vitro*, demonstrate that the *in vivo* potency of a toxicant cannot simply be deduced from its behavior in an *in vitro* system without careful validation of the model being used.

CONCLUSIONS

To characterize and understand mechanisms of multistep carcinogenesis *in vivo* and to

define carcinogenic risk from exposure to toxicants,

1) *in vitro* systems can provide useful information on the mechanisms of carcinogenesis and the carcinogenic risk leading to fewer and more-focused animal studies; however,

2) the relevance of *in vitro* endpoints or responses used in these analyses must be validated using *in vivo* determinations.

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In areas of research, Dr. Thomassen is investigating the mechanism of multistate progression to neoplasia in respiratory epithelial cells. The intent of this work has been (1) the identification of growth factors responsible for regulation of normal rat tracheal cell proliferation and differentiation and changes that occur with neoplastic or preneoplastic transformation, (2) a determination of the role of chemicals and radiation in the induction of neoplastic and preneoplastic transformation in rat tracheal epithelial cells, and (3) characterization of genes involved in human and rodent respiratory epithelial cell transformation. Dr. Thomassen is author or co-author of 31 publications.

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DEVELOPMENT OF A SHORT-TERM BIOASSAY TO ASSESS PULMONARY TOXICITY OF INHALED FIBERS

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ABSTRACT

This papers describes the development of a short-term inhalation bioassay using Kevlar® aramid fibrils. Five-day exposures to ultrafine Kevlar® fibrils elicited a transient granulocytic inflammatory response with concomitant increases in BAL fluid levels of alkaline phosphatase, NAG, LDH, and protein. Biochemical parameters returned to control levels at time intervals between 1 week and 1 month postexposure. Macrophage function in Kevlar®-exposed alveolar macrophages (as measured by morphology, in vitro phagocytic and chemotactic capacities) was not significantly different from sham controls at any time period. Cell labeling studies were carried out immediately after, 1 week and 1 month after a 5-day exposure. Increased pulmonary cell labeling was measured in terminal bronchiolar cells immediately after exposure but returned to control values 1 week later. Fiber clearance studies were carried out on Kevlar®-exposed, digested lung tissue. Fiber numbers increased 1 week postexposure, but cleared rapidly thereafter; fiber dimensions (lengths and diameters) decreased over a time period extending to 6 months postexposure, suggesting that Kevlar® fibers are soluble in lung tissue. This pattern is similar to results reported in fiberglass-exposed rats. Histopathologic analysis revealed no pathologic effects in the lungs of Kevlar®-exposed rats. The results indicate that the pulmonary toxicity of ultrafine Kevlar® fibrils is similar to effects transient measured in wollastonite and carbon fiber-exposed rats and differs significantly from results observed in crystalline silica or asbestos-exposed animals.

INTRODUCTION

There is a great need for the development of a rapid and reliable short-term bioassay to evaluate the pulmonary toxicity of new and untested fibers. Short-term tests are used to screen potentially toxic fibers for estimates of pulmonary toxicity, evaluations of mechanisms, and in dose-level setting for subchronic or chronic inhalation studies. It is likely that the data generated from short-term inhalation tests could be applied to setting dose levels for 90-day inhalation studies and thus obviate the need for costly and animal intensive 2-week or 28-day level-setting studies.

The development of our predictive screen is predicated on the hypothesis that the progression of particulate-induced, nononcogenic pulmonary disease is correlated with 4 interdependent general factors which include 1) cytotoxicity 2) inflammation, 3) alterations in macrophage clearance functions, and 4)

persistence of the material in the lung. In addition, we have begun to systematically evaluate mechanistic issues such as in vitro and in vivo fiber durability, fiber deposition, translocation, and clearance. An example of this application can be found in our studies with Kevlar® aramid fibrils.

These studies were undertaken to evaluate pulmonary mechanisms and the toxicity of inhaled Kevlar® fibers relative to other reference materials. Rats were exposed to ultrafine Kevlar® fibers for 5 days at gravimetric concentrations ranging from 2 - 13 mg/m³ (fiber numbers ranging from 600 - 1300 fibers/cc). Cells and fluids from groups of sham and fiber-exposed animals were recovered by bronchoalveolar lavage (BAL). Alkaline phosphatase, lactate dehydrogenase (LDH), protein, and N-acetyl glucosaminidase (NAG) values were measured in BAL fluids at several time points postexposure. Cells were identified, counted and evaluated for viability. Alveolar macrophages (AM) were cultured and studied for morphology, chemotaxis, and phagocytosis by scanning electron microscopy. The lungs of additional exposed animals were processed for cell labeling and lung clearance studies, fiber dimensions (from digested lung tissue), histopathology and transmission electron microscopy.

MATERIALS AND METHODS

General Experimental Design

Groups of male Crl:CD®BR rats (8 weeks old, Charles River Breeding Laboratories, Kingston, New York) were used to assess the pulmonary effects of short-term, high-dose aerosol exposures to Kevlar® fibrils. Rats were exposed 6 hr/day for 5 days to Kevlar® concentrations ranging from 600 - 1344 f/cc (2 - 13 mg/m³). Following exposures, fiber-exposed animals and aged-matched sham controls were evaluated at several time points postexposure (i.e., 0, 24, or 72 hrs, 1 week, 1, 3 or 6 months).

Fiber Preparations

Ultrafine Kevlar® para-aramid fibrils were supplied by Du Pont Fibers. A special preparation of ultrafine, respirable-sized Kevlar® fibers (heretofore referred to as fibrils) which had been prepared for the 2-year inhalation study (1) was utilized for this study.

Inhalation Exposure

Dust generation techniques were similar to those described by Lee and colleagues (1) and are described in detail elswhere (2). Briefly, atmospheres of ultrafine Kevlar® fibrils were generated with a K-tron® bin feeder (K-tron Co., Glassboro, NJ) equipped with twin screws. Baffles were inserted into the generation apparatus and served to increase the respirability of the sample. Kevlar® fibrils were metered into a plastic funnel connected to a cyclone where high pressure air transferred the test material into a micro-jet apparatus (Micro-jet, Fluid Energy Co., Hatfield, PA). This high pressure air-impingement device was utilized to separate ultrafine Kevlar® fibrils from the larger fiber clumps. Kevlar® fibrils were then drawn through another cyclone and then into the chamber. Chamber concentrations of fibrils were maintained by controlling the dust-feed rate into the generation apparatus, or by varying the air-flow rate. For gravimetric analysis, samples of atmospheric Kevlar® were taken from the animal breathing zone at approximately 30-minute intervals by drawing calibrated volumes of chamber atmosphere through preweighed glass-fiber filters. Filters were weighed on a Cahn 26 automatic electrobalance. The atmospheric concentrations of Kevlar® were determined from

filter weight differentials before and after sampling. Particle size measurements of airborne fibers in the test chamber were determined with a Sierra casade impactor and reported as mass median aerodynamic diameter (MMAD). Fiber counts were carried out according to the NIOSH 7400B method.

In addition to gravimetric concentration, fiber numbers in the aerosol, and mass median diameter measurements, fiber size dimensions were analyzed by a scanning electron microscopic (SEM) method. Airborne filters (used for determining fiber concentrations) were prepared for SEM as described previously (3). The filters were mounted onto carbon stubs, gold-sputtered, and studied in a calibrated JEOL 840 electron microscope. Fiber dimensions were quantified using a technique that allowed random selection of fibrils by first selecting a field of view at low magnification to avoid bias. All selected areas were then studied at higher magnification (i.e., 2000x and/or 5000x). Numerous high magnification SEM micrographs were taken per field of view. The dimensions (lengths and diameters) of at least 160 fibers/filter were then measured on the micrographs using a ruler. For fiber classification purposes in these SEM studies, fibers were defined as having an aspect ratio of 3:1 (length:width) and a minimum length of 4 μm. Following tabulation of the data, a count median length (CML) and count median diameter (CMD) for the fiber aerosol were determined.

Pulmonary Lavage and Biochemical Assays on Bronchoalveolar Lavage Fluid

Bronchoalveolar lavage procedures were conducted according to methods previously described (2). Lavaged fluids recovered from sham and dust-exposed rats were centrifuged and concentrated for biochemical studies. The cell pellet was resuspended in Eagles Minimal Essential Medium. The methods of quantitation of cell numbers, viabilites and differential counts have been previously described. All biochemical assays were performed on concentrated BAL fluids at 30°C, using a semi-automated clinical chemical analyzer. Methods for quantifying lactate dehydrogenase (LDH), alkaline phosphatase (ALP), protein, and N-acetyl-β-glucosaminidase (NAG) in BAL fluids have been previously reported (2).

Pulmonary Cell Labeling Studies

BrdU cell labeling methods have been previously described (2). Groups of Kevlar® fiber and sham-exposed rats were pulsed immediately after 5-day exposure, as well as 1 week or 1 month postexposure with an intraperitoneal injection of 5-bromo-2'deoxyuridine (BrdU) dissolved in a 0.5 N sodium bicarbonate buffer solution at a dose of 100 mg/kg body weight. The animals were sacrificed 2 hr later by pentobarbital injection.

Recovery of Kevlar® Fibrils from Digested Lung Tissue

Kevlar® fibers were recovered from the lungs of exposed rats according to methods previously reported (2). Briefly, fixed lung tissue was digested in an 11% KOH solution (in ethanol and water. The numbers of fibers/area of filter were counted by phase contrast light microscopy using the NIOSH 7400B counting method (NIOSH Manual of Analytical Methods). For fiber classification purposes, fibers were defined as particulate materials with an aspect ratio of 3:1 (length:width). In addition, only fibers $\geq$ 5 μm in length were counted. Fiber dimensional analysis was carried out by scanning electron microscopy (SEM); fiber $\geq$ 4 μm in length were included for dimensional analysis.

RESULTS AND CONCLUSIONS

Chamber Atmosphere Analysis

The exposure generation data for aerosolized Kevlar® fibrils are summarized in Table 1.

Analyses of Cellular Constitutents in BAL

Exposures to Kevlar® fibrils did not alter the total numbers of cells recovered by lung lavage. The viability of cells recovered in fiber or sham-exposed rats was greater than 94% at all time periods.

Cell differential analyses of lavaged cells recovered from Kevlar®-exposed rats demonstrated that fiber inhalation produced an early but transient pulmonary inflammatory response, as evidenced by increased numbers of neutrophils and biomarkers (i.e., LDH, NAG, and protein) in BAL fluids (Table 2). This effect was measured through a 1-week postexposure period but was short-lived since neutrophil numbers were not significantly different from sham control values at the 1 month postexposure period, or at any other subsequent time period.

Cell Labeling Studies

No significant differences in the labeling index of lung parenchymal cells were detected between Kevlar®-exposed rats and their corresponding sham controls at any time period (Table 3). Increased BrdU labeling of terminal bronchiolar cells in Kevlar®-exposed rats was measured immediately after exposure. However, no significant differences were measured at 1 week or 1 month postexposure, indicating that this effect was transient. Histopathologic analysis at 3 months post Kevlar® exposure indicated that no pulmonary lesions were detected in fiber or sham-exposed control rats.

Fiber Clearance Studies

Clearance studies demonstrated a transient increase in the numbers of retained Kevlar® fibrils at 1 week postexposure, with rapid clearance of fibers thereafter (data not shown). The estimated clearance half-time was 90 days. The finding of increased numbers of retained fibers at 1 week postexposure in Kevlar®-exposed rats may be related to transverse cleaving of the fibrils. It is likely that fiber shortening during the first week after exposure could account for this apparent increase. Mean fiber lengths recovered from digested lung tissue decreased from 12.5 µm to 7.5 µm, measured over a 6-month postexposure period. Similarly, mean fiber diameters decreased from 0.33 µm to 0.23 µm over the 6-month period (Table 4). These results suggest that Kevlar® fibrils are shortened in the lungs of exposed rats. In this regard, the pulmonary clearance mechanisms associated with inhaled Kevlar® fibrils are different from those associated with chrysotile and crocidolite asbestos, wherein the mean lengths of inhaled fibers are progressively increased over time (4-5).

A short-term inhalation bioassay is being developed to evaluate the potential for inhaled fibers or fibers to produce lung injury, i.e., pulmonary fibrosis. The estblishment of this predictive screen is grounded on the detection of a series of biomarkers which may be predictive of the progression of fiber-induced pulmonary injury. In previous studies, we have assessed the efficacy of this short-term inhalation screen by exposing rats to several

concentrations of numerous reference particulate or fibrous materials. These include known fibrogenic dusts such as α-quartz silica (6) and crocidolite asbestos (7), as well as materials with minimal or moderate biological activity such as titanium dioxide and carbonyl iron particles (6), or carbon fibers (8). Short-term exposures to silica or crocidolite asbestos in rats produced persistent pulmonary inflammatory (neutrophilic) responses. In contrast, exposures to Kevlar® fibrils caused only transient inflammatory effects. The findings here support the conclusion that acute inhalation of fibers with low durability such as Kevlar® are likely to produce only short-term pulmonary effects while inhalation of fibrogenic dusts such as silica or durable fibers such as crocidolite produce sustained pulmonary inflammatory effects along with consistently elevated indicators of cytotoxicity and consequent development of pulmonary fibrosis.

ACKNOWLEDGMENTS

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TABLE 1.

KEVLAR FIBRIL EXPOSURE DATA AND INHALED DOSE

Experiment #1*#@

DURATION	MMAD (δg)	(mg/m³)	FIBER #/cc	DOSE(f/g)	CML	CMD
5 DAYS	4.5 μm (2.7)	4.4	1073	3.2 x 10^6	9.9 μm	0.3 μm
5 DAYS	3.4 μm (2.7)	8.5	1344	3.5 x 10^6	9.9 μm	0.3 μm
			Experiment #2@			
5 DAYS	3.2 μm (2.9)	2.9	613	1.4 x 10^6	10.0 μm	0.3 μm
5 DAYS	4.7 μm (3.2)	11.1	877	1.3 x 10^6	10.0 μm	0.3 μm

* Used for bronchoalveolar lavage studies
# Used for cell labeling studies
@ Used for fiber clearance/retention studies
CML = Count median Length
CMD = Count median diameter

TABLE 2.

PULMONARY INFLAMMATION RESPONSES IN KEVLAR®-EXPOSED RATS

Numbers of Lavaged Neutrophils

Time After 5-Day Exposure

	0H	24H	1WK	1M	3M
SHAM	8.0 X10^3	7.8 X10^3	7.0 X10^3	7.3 X10^3	8.6 X10^3
KEVLAR	4.1 X10^5 *	4.4 X10^5 *	3.3 X10^5 *	2.6 X10^4	8.7 X10^3

BAL PROTEIN, LDH, AND NAG VALUES

(% of Sham Control)

Time After 5-Day Exposure

	LDH		
SHAM	100	100	100
KEVLAR II (1073 f/cc)	426	416	111
KEVLAR III (1344 f/cc)	375	512	148
	Protein		
SHAM	100	100	100
KEVLAR II	193	227	115
KEVLAR III	156	304	126
	N-acetyl-β-glucosaminidase		
SHAM	100	100	100
KEVLAR II	300	186	80
KEVLAR III	236	189	98

TABLE 3.

PULMONARY CELL LABELING RESPONSES IN KEVLAR®-EXPOSED RATS

Numbers of Lavaged Neutrophils

PULMONARY CELL LABELING
% LABELED CELLS

Time After 5-Day Exposure

Terminal Bronchiolar Cells

	0H	1 Week	1 Month
SHAM	0.8 ± 0.2	0.9 ± 0.1	1.1 ± 0.1
KEVLAR®	2.3 ± 0.4*	1.3 ± 0.1	1.2 ± 0.1

Pulmonary Parenchymal Cells

SHAM	1.0 ± 0.1	1.1 ± 0.2	1.1 ± 0.1
KEVLAR®	1.4 ± 0.2	1.1 ± 0.6	1.0 ± 0.1

* $p < 0.05$

TABLE 4.

KEVLAR® FIBRIL DIMENSIONAL DATA

Time After 5-Day Exposure

	0H	72H	1M	3M	6M
			Mean Lengths		
KEVLAR®	12.5 μm ± 2	11.1 μm ± 5	9.0 μm ± 2	8.1 μm ± 2	7.45 μm ± 3
			Mean Diameters		
KEVLAR®	0.33 μm ± 0.03	0.30 μm ± 0.06	0.295 μm ± 0.04	0.27 μm ± 0.09	0.23 μm ± 0.02 μm

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IN VITRO TOXICITY OF REFRACTORY CERAMIC FIBERS TO CHINESE HAMSTER OVARY CELLS IN CULTURE

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Abstract

The toxic effects of four different compositions of refactory ceramic fibers (RCF) were determined using Chinese hamster ovary (CHO) cells grown in culture. The RCF's were the same size-selected fibers used in recent chronic rodent inhalation studies which assessed in vivo toxicity/oncogenicity of RCF's. The in vitro toxic endpoints--proliferation, cloning efficiency, and abnormal nuclei induction--were compared with in vivo endpoints. The fibers most toxic in vitro were also most toxic in the chronic animal inhalation studies. A direct relationship was also observed, both in vitro and in vivo, between average fiber length and severity of toxic effect.

Introduction

Awareness of the human health hazards associated with asbestos exposure has led to the development of many new man-made and naturally occurring fibers to be used as asbestos substitutes. The importance of testing all fibrous products for their pathogenic potential is generally recognized, however many fibrous materials currently in use and in the product-development stage have not been evaluated. Animal inhalation testing is currently the only accepted laboratory model for determining the potential human health hazard of respirable particles[1]. Because this form of testing is time-consuming and expensive, short-term tests are needed to screen fibers for their potential toxicity.

Any short-term test system proposed as a means of screening fibers for their toxicologic potential must be validated by demonstrating that the effects observed using the screening system correlate with human epidemiological data and/or animal inhalation data. A recent chronic rodent inhalation study of the toxicity and tumorigenicity of refractory ceramic fibers[2] afforded the opportunity to make these correlations. Conducting in vitro research in parallel with in vivo studies also provides an additional perspective on the toxic mechanisms at the cellular level. Thus, the present study was conducted with these two goals in mind: 1) to develop and validate in vitro assays that could be useful as a part of a battery of short term fiber toxicology screening tests; and 2) to contribute to a more complete understanding of the mechanisms of fiber-cell interactions.

Materials and Methods

Test Fibers. Four different compositions of refractory ceramic test fibers were obtained from the TIMA fiber repository (c/o Manville Sales Corporation, Attn. T.W.Hesterberg, P.O. Box 5108, Denver,CO. 80217-5108) RCF1 (kaolin), RCF2 (zirconia), RCF3 (high purity) and RCF4 (after service). These are size-selected, respirable test fibers that were used in rodent inhalation studies [1,2,3]. Standard UICC (International Union Against Cancer) crocidolite (CD) and UICC chrysotile (CH) asbestos were used. Fiber data (Table I) were analyzed using scanning electron microscopy (SEM) for the RCFs and CD and transmission electron microscopy (TEM) for CH.

TABLE 1.

Physical Characterization of Fibers Using Electron Microscopy

Test Fiber		No. Fibers/ng	Fiber Dimensions, μm	
Ref. ID	Fiber Type		Avg.Diameter ± std. dev.	Avg.Length ± std. dev.
RCF1	Kaolin	4.7	1.03 ± 0.73	21.5 ± 16.12
RCF2	Zirconia	4.6	1.11 ± 0.82	16.7 ± 15.03
RCF3	High Purity	3.1	1.22 ± 0.98	24.3 ± 18.82
RCF4	Aft. Serv.	6.3	1.43 ± 0.79	9.2 ± 7.08
Asbestos				
CD	UICC Crocidolite	2400	0.21 ± 0.12	1.81 ± 1.94
CH	UICC Chyrsotile	4400	0.12 ± 0.07	1.65 ± 1.83

Cell Cultures and In Vitro Assays. Frozen CHO-K1 cells were obtained from American Type Tissue Collection (Rockville, MD) and were thawed and plated in tissue culture flasks with complete medium (CM) consisting of Ham's F12 supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 1 ml Fungi-Bact solution/100 ml medium (all from Irvine, Santa Ana, CA). To create a large bank of uniform stock cells for experiments, cultures were incubated (37°C, 5% CO_2 and 100% humidity) and allowed to replicate for several days (three-four doublings), then harvested with 0.05% Trypsin and 0.02% EDTA in Hanks balanced salt solution (Irvine). The stock cells were then frozen at -80°C and later transfered to liquid nitrogen for storage. For experiments, stock cells were thawed and plated in CM and allowed to grow for 2-4 days.

Inhibition of Cell Proliferation (ICP) Assay. For ICP assays, 60 mm culture dishes were seeded with 100,000 cells in 5 ml CM/dish. After 24 hrs. incubation, fibers were added to cultures in one ml CM/dish. Dishes were gently agitated to disperse fibers over the bottom. Negative control cultures received 1 ml CM/dish. Each exposure group was set up in triplicate. After three days exposure, incubated as described above, cells were harvested using trypsin (as described above) and counted using a Coulter counter (Coulter Electronics, Hialeah, Florida). Relative proliferation was determined by dividing the number of cells present in each exposed culture by the number of cells present in negative control cultures.

Colony Forming Efficiency (CFE). For the CFE assay, 60 mm culture dishes were seeded with 200 cells in 5 ml complete medium and exposed as described for ICP above. Each exposure was set up in triplicate. After 5 days exposure, colonies were stained with 0.4% w/v Giemsa in buffered methanol (Sigma). Colonies with >10 cells were counted using a stereoscope at low power. CFE was determined by dividing the number of colonies in each exposed culture by the number of colonies in unexposed cultures.

Micronuclei (MN) and Polynucleus (PN) Induction. For these assays, cultures were prepared as described for ICP above. After two days exposure, culture dishes were fixed with methanol/acetic acid (3:1, v/v) and stained with 0.01% acridine orange (from Sigma; as described in Clark[4]). Using a microscope with epifluorescence, the percentage of cells containing micronuclei (MN) and/or polynuclei (PN) was determined for each culture dish. An MN was defined as a nucleus that appeared to be less than one-half the size of the normal-sized nuclei; PN was defined as a cell having bi- or multiple nuclei or a lobed nucleus. A minimum of 100 cells/dish and 2-3 dishes/exposure group were scored for (1) presence of MN but no other visible nuclear abnormalities, (2) presence of both MN and PN, or (3) PN but without micronuclei. Calculations included the percentage of cells with MN (including both uninucleate and polynucleate cells), the percentage of PN (with or without MN), and the percentage of cells with either type of nuclear abnormality (NA), ie., having MN and/or PN.

Results

Results of the three in vitro assays are shown (Figures 1-3). These data show the same relative toxicities for the four RCFs when concentration is based on μg/cm^2. In each case RCF4 is the least toxic, RCF2 is intermediate, and RCF1 and RCF3 are the most toxic. In all three assays, CD was more toxic than the RCFs; in the ICP (Fig. 1) and MNI (Fig. 3) assays, CH was more toxic than CD (CH was not included in the CFE assays.)

Although some of the error bars (SEM, standard error of the means) overlap, in each individual test performed with these fibers (more than 14 independent tests in all), the same relative toxicities resulted.

Fig.1. Inhibition of Cell Proliferation

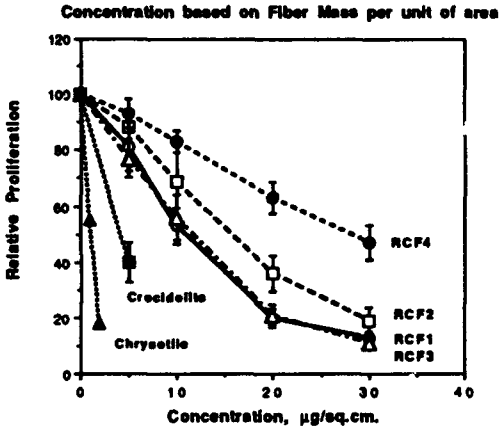


Fig. 2. Colony Forming Efficiency

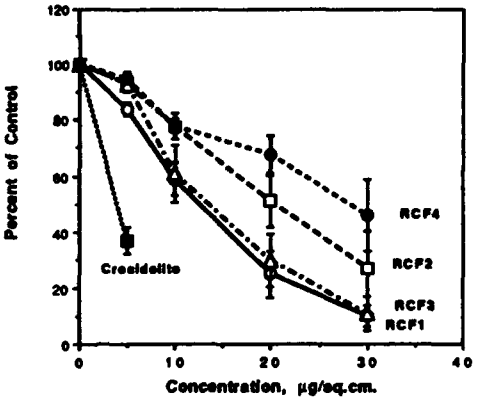


Figure 3. Induction of Nuclear Abnormalities

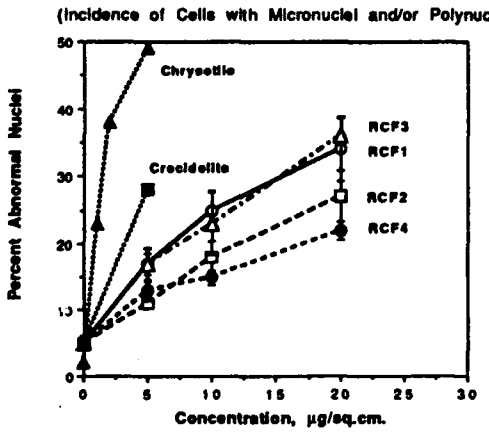
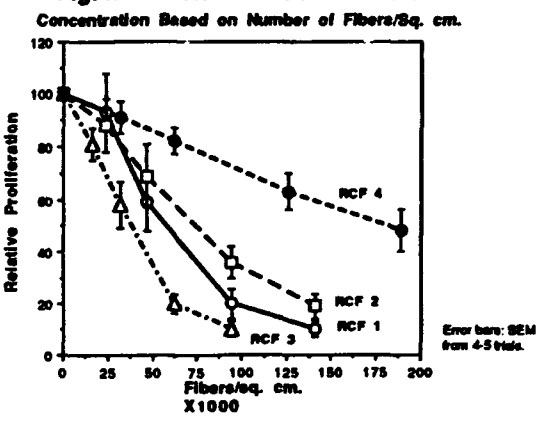


Fig.4. Inhibition of Proliferation



When the concentration of the RCFs is expressed as number of fibers/cm^2 (Figure 4), the similar relative toxicities of the different RCFs is observed, although the spread between RCF1 and RCF3 is increased. Data from the asbestos types are not plotted in this manner since both the length and diameter are an order of magnitude less than with RCF fibers, making a fiber-to-fiber comparison inappropriate.

CHO cells in culture exposed to RCF2 are seen in Figure 5. Nuclear abnormalities (NA) observed in all the fiber-exposed cultures appeared qualitatively similar. Some examples of NA include: PN which appear to be clearly more than one nucleus as well as PN which appear to be bi-or multi-lobed; micronuclei with one normal-appearing nucleus or with PN. The relationship between intracellular fibers and nuclear distortion was unmistakable in all the fiber exposed cultures and was especially apparent when both PCOM and fluorescent microscopy were used to view the cells. Both wholly and partially internalized fibers were seen.

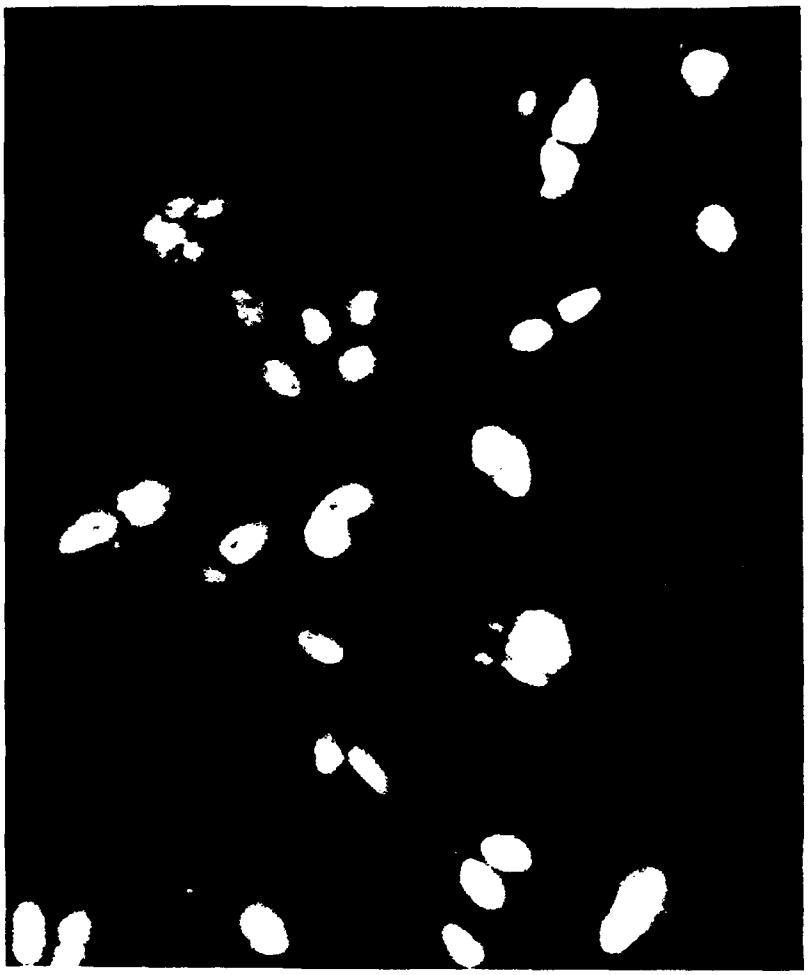


Figure 5

CHO Cells exposed to RCF2, 800x magnification. Cells with both normal and abnormal nuclei are visible. Note the micronuclei and polynuclei. Acridine Orange stain, photographed with flourescent light.

In the fiber-exposed cultures, the incidence of both types of NA (percentage of cells with MN and/or PN), was strongly concentration-dependent, whereas the incidence of MN alone, although elevated above background levels, was weakly concentration-dependent. CH at 5 μg/cm^2 induced NA in 49% of the cells; CD at 5 μg/cm^2 induced NA in 28% of the cells. The NA incidence for RCFs at 20 μg/cm^2 ranged from 20-40% (Fig. 3).

TABLE 2

Comparison of In Vitro and In Vivo Toxic Endpoints

Exposure Group	Toxic Endpoints				
	In Vitro LC50, µg/sq.cm.		In Vivo		
	ICP	CFE	Wagner Score (6 Months)	% with Lung Tumors	% with Mesotheliomas
Negative Control	-	-	1.0	1.6	0.0
RCF1	13	10	4.0	13.0	1.5
RCF2	17	18	3.0	6.9	2.3
RCF3	13	12	4.0	14.6	1.5
RCF4	30	22	2.7	3.2	0.8

In Table 2 above the toxic endpoints of the in vitro studies are compared with those of the animal inhalation studies using the same RCF test fibers.The LC-50 is the in vitro concentration which resulted in 50% as many cells or colonies in fiber-treated cultures as in unexposed control cultures. The higher the LC-50, the lower the in vitro toxicity of the fiber. Wagner grades were used to quantitate the lung pathology observed in the chronic inhalation study of RCFs: 1 indicates normal lung; 2 is macrophage infiltration; 3 is inflammation and bronchiolization; 4 is minimal but irreversible fibrosis; 5, 6, 7, and 8 are increasingly severe fibrosis[3]. In the animal inhalation studies, three to six animals per group were scored every three to six months during the 24 month exposure to fiber aerosol and at the termination of the lifetime study. Only the six month scores are given for comparison here, because all fiber-exposed lung scores plateaued near grade 4 soon after the 6 month time point (air control lungs remained at level 1). The columns on the right in Table 2 indicate the percentage of animals which developed lung tumors and mesotheliomas by the end of the RCF inhalation study . The relative toxicity of the different RCFs seen in vitro correlates with the fibrosis and lung tumors observed in vivo: RCF4 is least toxic, RCF2 is intermediate, and RCF1 and RCF3 are more toxic. Mesothelioma results vary slightly from this pattern in that RCF2 induced more tumors than RCF1 and 3, but RCF4 was still least toxic.

Discussion

The present study provides several pieces of information that contribute toward the goals of this project: to develop short-term screening tests that can be used to assess the toxicologic potential of fibrous dusts and to increase understanding of the mechanisms of fiber toxicity at the cellular level. First, the CHO cell test system is highly responsive to fiber exposure in all three parameters investigated; concentration-dependent reductions in cell proliferation and cell colony formation as well as increases in nuclear abnormalities were observed following exposure to each of the test fibers. Secondly, specificity is evident in this model, in the consistent relative response of these cells to the six different fibers. Thirdly, cellular uptake of fibers and alterations of nuclear morphology are clearly major CHO cell responses. The observed fiber-induced nuclear abnormalities fell into two basic categories (MN and PN) and the incidence of each was consistent for the six fibers in three or more separate assays. The mechanism of MN induction may be different from that of PN. MN are believed to form when chromosomes or fragments are separated from the migrating masses of replicate chromosomes during mitosis. Previous studies have shown a correlation between incidence of micronuclei and aneuploidy in three different cell lines (CHO cells[5], Syrian hamster embryo [SHE] cells[6]; human lymphocytes[7]). PN may result from the failure of cytokinesis following mitosis. And finally, the incidence of total nuclear abnormalities was approximately the inverse of the proliferation curves, indicating that disruption of mitosis may be the major or immediate

cytotoxic effect, rather than the disruption of cytoplasmic metabolic processes. However, validation of this hypothesis would require cell viability testing.

Differences in the length of the four RCFs can probably account for the differences in both the in vitro cytotoxicity and the in vivo lung pathology observed in the animal inhalation study. A comparison of Tables 1 and 2 demonstrates that the fiber length parallels toxicity. Many previous in vitro studies also report a relationship between fiber length and cytotoxicity. Brown et al.[8] demonstrated that glass fibers were more toxic to cultured macrophages and to V79-4 cells when average fiber length was greater than 10μm; Lipkin et al.[9] observed a relationship between fiber length and the reduction of cell proliferation in cultures of P388D1 cells exposed to several populations of glass fibers; Tilkes and Beck[10,11,12] showed a direct relationship between fiber length and LDH release (macrophages and ascites) and reduction in proliferation (ascites) in cell cultures exposed to fibrous glass.

A mechanism to explain the relationship between fiber length and cytotoxicity could be related to cell diameter. For example, when the flattened, adherent cell becomes spherical as it enters metaphase prior to cell division, internalized fibers that are longer than the diameter of the metaphase cell might puncture the cell, causing cell death. Fiber length has also been shown to be important in the induction of cytogenetic effects by internalized fibers[13] and in the transformation of cultured cells[14]. In a study that demonstrated direct interaction between intracellular asbestos fibers and migrating chromosomes during anaphase in cultured SHE cells, Hesterberg and Barrett[15] suggested that long fibers are more easily entangled in the migrating chromosomes or spindle apparatus than are short fibers.

CONCLUSIONS

The concordance between in vitro and in vivo toxic endpoints in the RCF studies is encouraging. However, more research is needed to determine which in vitro test systems and procedures will best correlate with animal inhalation studies and human epidemiology. Again, fibers tested in long-term animal inhalation studies will be needed for validation of the sensitivity and specificity of these short-term assays. Such fibers should be well characterized chemically and physically (length, width, number of fibers/unit mass). Furthermore, the test fibers should include a range of demonstrated in vivo toxicities, from non-toxic to strongly toxic. In vitro testing should include other cell types and other in vitro parameters (ie., durability, cytotoxicity and mutagenesis).

Several parameters of in vivo pathogenesis cannot at this time be measured in vitro, eg., lung deposition, lung clearance, and long-term biopersistence. Some work has been done in the third area, by incubating fibers in flow-through systems using synthetic physiological solutions[16,17]. However, such systems do not contain all of the factors that might be important in the biopersistence of fibers in the lung, such as enzymes, lysosomal secretions, and mechanical removal by phagocytic cells. These limitations might be overcome by including short-term animal inhalation studies in the battery of toxicity screening tests.

A more detailed report of this study can be found in *Toxicology In Vitro*[18].

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Session V: *In vitro* and Other Alternatives in Environmental Toxicology

Co-Chairs: Drs. Randall S. Wentsel and James J. Murphy

RANDALL S. WENTSEL, Ph.D.

Dr. Randall S. Wentsel is the Chief of the Environmental Toxicology Branch, Toxicology Division at U.S. Army Chemical Research Development and Engineering Center, Aberdeen Proving Ground, MD.

Dr. Wentsel received his Ph.D. In Environmental Toxicology, Health/Environmental from the Purdue University. His research areas have included the impact of heavy metals on benthic organisms in aquatic systems, sediment toxicology, terrestrial toxicology, and the transport and transformation of chemicals in the environment. Recently he has focused on ecological risk assessment. Dr. Wentsel is active in the Society of Environmental Toxicology and Chemistry. He was the program chairperson for the 1990 Annual Meeting.

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Dr. Murphy is a toxicologist in the Office of Pollution Prevention and Toxics (formerly the Office of Toxic Substances) at the U.S. Environmental Protection Agency Headquarters in Washington, DC. He has also served at EPA in the Office of Pesticide Programs and the Office of Drinking Water. Before joining EPA, he was a toxicologist and criteria-document manager in Stanford Research Institute's Center for Occupational and Environmental Safety and Health, preparing criteria documents for the National Institute for Occupational Safety and Health. He earned a bachelor's degree in chemistry at Lafayette College, a master's degree in clinical psychology at St. John's University in New York, and a Ph.D. in physiology and biophysics at Temple University. He has been a diplomate of the American Board of Toxicology and its specialty sections in immunotoxicology, inhalation toxicology, and risk assessment. He is also a member of the Society for Risk Assessment, the Association of Government Toxicologists, and the International Society for the Study of Xenobiotics. He has served on expert panels of the multinational negotiating mutually acceptable testing guidelines for acute lethality, skin and eye irritation, skin sensitization, and toxicokinetics. Dr. Murphy has been an invited speaker for a number of organizations, including the Society of Comparative Ophthalmology.

OVERVIEW OF *IN VITRO* AND OTHER ALTERNATIVES IN ENVIRONMENTAL TOXICOLOGY

JAMES J. MURPHY, Ph.D.
UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

I'm very pleased to have been invited to participate in this symposium. Although the Environmental Protection Agency has long been interested in testing alternatives, any remarks I may make will be my own private opinions, and not necessarily reflect the policies of the Agency.

We have a consciousness-expanding program for you throughout this symposium. Traditionally, we tend to think of mammals, such as rats, mice, rabbits or guinea pigs, when we think of testing for safety- or hazard- assessment. Throughout this symposium we will expand our horizons with a variety of less traditional models. For example, we will see how macrophages *in vitro* can yield useful information. We will see how worms may be substituted for rodents in testing. We will see an approach to assessment of developmental toxicity using frog embryos instead of mammals. Fish have been used for carcinogenicity screening as well as for assessing effects on aquatic ecosystems.

One area in which *in vitro* testing offers particular promise is in simplifying experimental systems to make it easier to zoom in on and elucidate molecular mechanisms of action. Following the analogy of the microscope, one can appreciate that a high-power look at the molecular level implies that many iterations will be required to build up a panoramic overview of what is happening. Therefore, *in vitro* tests for safety evaluation are generally pursued in the context of batteries of several tests. Although a single *in vitro* test may be much less expensive than an intact-animal test, the difference in cost is not so great between a battery of *in vitro* tests and an intact-animal test.

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POTENTIAL FOR INTERSPECIES EXTRAPOLATION OF MACROPHAGE CHEMILUMINESCENCE DATA FROM IMMUNOTOXICOLOGY STUDIES

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ABSTRACT

Organisms at all phyletic levels appear to experience immunomodulation after exposure to sublethal concentrations of certain environmental chemical stressors. A portion of the observed immunological dysfunction may arise from chemical-macrophage interactions. Normal macrophage functions must be maintained to insure integrity of the immune system because of the roles of these cells in antimicrobial defense and in cytokine-mediated immune regulation. Macrophage-like cells have been highly conserved during the evolutionary process; therefore, it is likely that immunotoxicological data from macrophage studies probably have high potential for interspecies extrapolation. Similarities between the modulation of phagocytic antibacterial mechanisms by xenobiotics in mollusks and mammals are described in this paper.

INTRODUCTION

Virtually all animals so far studied contain macrophages or macrophage-like cells in one or more of their body compartments. These cells are typically phagocytic and serve to remove and sequester microbes and other nonself materials from the blood or other host tissues. In this paper, the phagocytes that exist free in the blood, hemolymph and body cavity fluids of mammals and invertebrates will be compared as to form and function. The aim is to describe the numerous similarities between these cells from phyletically diverse species with regard to the mechanisms by which they establish resistance to infectious disease. In keeping with the theme of current concepts and approaches on animal test alternatives, two hypotheses are proposed: invertebrate macrophages emulate the chemically-induced immunomodulatory behavior characteristic of mammalian macrophages and invertebrate macrophages lend themselves well to in vitro

immunotoxicity testing protocols because of their quantifiable activities in short-term, primary culture.

Phagocytic blood cells, macrophages and other cells involved in antimicrobial activities generally employ intracellular hydrolytic enzymes and reactive oxygen intermediates (ROIs) to mediate cytotoxicity. Clearly, interference with these mechanisms resulting from exposure to chemical stressors could compromise an organism's resistance to infectious disease. Appropriate stimulation of cell surface receptors and/or ingestion of bacteria, or other foreign material, can initiate increased metabolic activity (the respiratory burst) and the production of the superoxide anion (O_2^-).[1-4] Superoxide is the precursor of a series of other ROIs such as hydrogen peroxide (H_2O_2), hydroxyl radical, and singlet oxygen. Both O_2^- and H_2O_2 have direct cytotoxic activities[5], and H_2O_2 plus myeloperoxide and chloride ions can form one of the most effective antibacterial systems of leukocytes.[6] In this study, phagocytically stimulated cellular chemiluminescence, augmented by the presence of 5-amino-2,3dihydro-1,4-phthalazinedione (luminol), was quantified as an indication of the production of ROIs. Oxidation of luminol by ROI produced during the respiratory burst results in the generation of excited aminophthalate anions which release photons during relaxation to the ground state.[7] This chemiluminescence (CL) was accurately quantified in a liquid scintillation counter adapted for single photon monitoring.

Immunotoxicological studies with higher animals indicate that certain chemicals possess specific immunomodulatory activity toward macrophages.[8-11] Identification of these chemicals is a priority because changes in macrophage responsiveness could have profound effects on the health of the host. Macrophage CL has been suggested as a tier one screening assay for identifying immunomodulatory chemicals, using cells from laboratory rodents.[12] Bivalve mollusks have large numbers of circulating macrophage-like cells that also actively produce luminol augmented CL.[13-14] We have been investigating chemically induced immunosuppression in hemocytes of the American oyster (*Crassostrea virginica*) as part of our interest in developing animal test alternatives and in evaluating the impact of aquatic pollutants on immunocompentency of estuarine species.

METHODS

Hemolymph samples were withdrawn from the adductor muscle and added to plastic culture dishes. Most hemocytes adhered tightly to the plates and 30 min. later the supernatant (serum and nonadherent hemocytes) were replaced by oyster cell support medium (CSM). Cell support medium contained 5% fetal calf serum, 0.5% antibiotic-antimycotic solution and 1 mg/ml glucose in filter-sterilized ambient estuarine water. After two hours incubation, the cells began to lose adherence and could be gently washed off the substrate with CSM. The cells were washed, counted and resuspended in CSM. Scintillation pony vials were prepared; each vial contained 2 x 10^6 hemocytes in CSM, which were exposed to toxicant (in this

case particulate brass dust) for 1 and/or 20 hours in the dark. All following steps were carried out under dim red illumination in order to reduce background, cell unrelated, CL. After incubation/exposure, luminol (100 μM final concentration) was added, and the CL activity of the resting cells recorded for 5-10 min. Then heat-killed yeast cells (20 yeast cells:1 hemocyte) were added to provide phagocytic stimulation and the resultant induced CL continuously recorded for 60 min. CL determinations were carried out in a scintillation counter modified for single photon counting. Aliquots of all hemocyte samples were tested for viability by trypan blue exclusion immediately prior to CL analysis.

RESULTS AND DISCUSSION

Representative results of dose-dependent inhibition of hemocyte CL by exposure to brass dust are represented in Figures 1 and 2. In these figures, aliquots of a single hemocyte pool were exposed to the agent for 1 or 20 h; mean cell viability ranged from ~80-90% for 1 h samples, and from ~75-90% for the 20 h samples. However, the CL responses of these cells were markedly diminished by brass exposure, as shown in the figures.

FIGURE 1

Effect of One-Hour Exposure to Particulate Brass on Oyster Hemocyte CL.

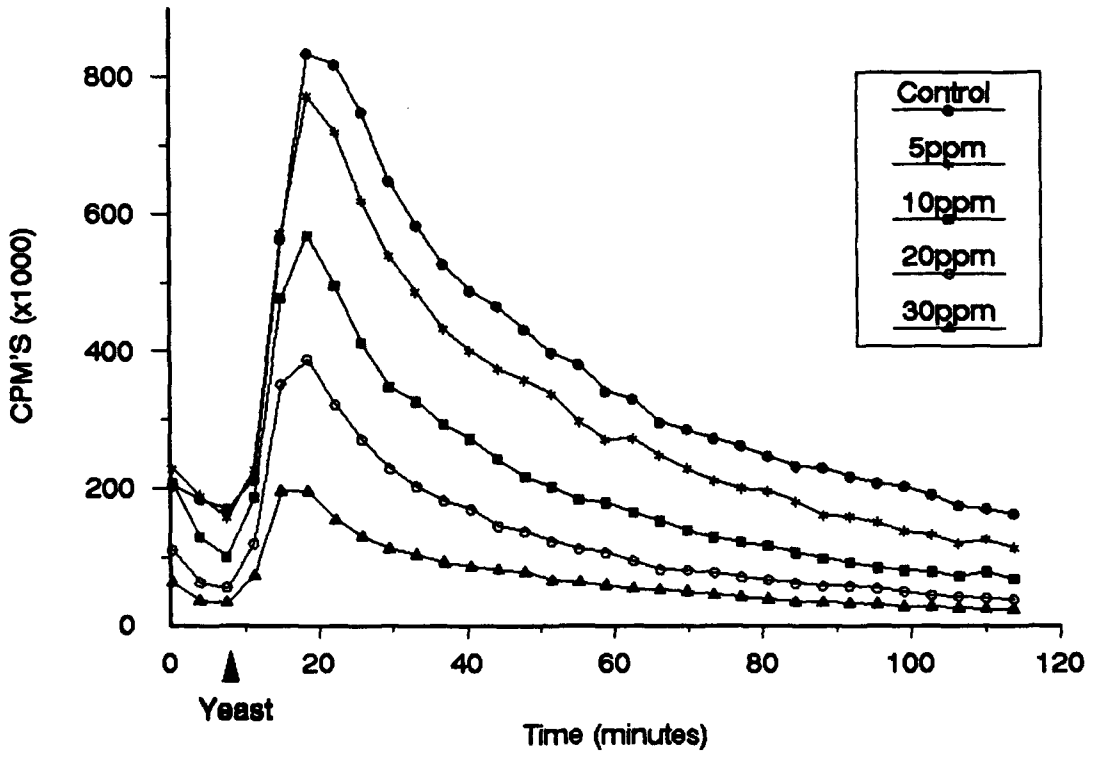
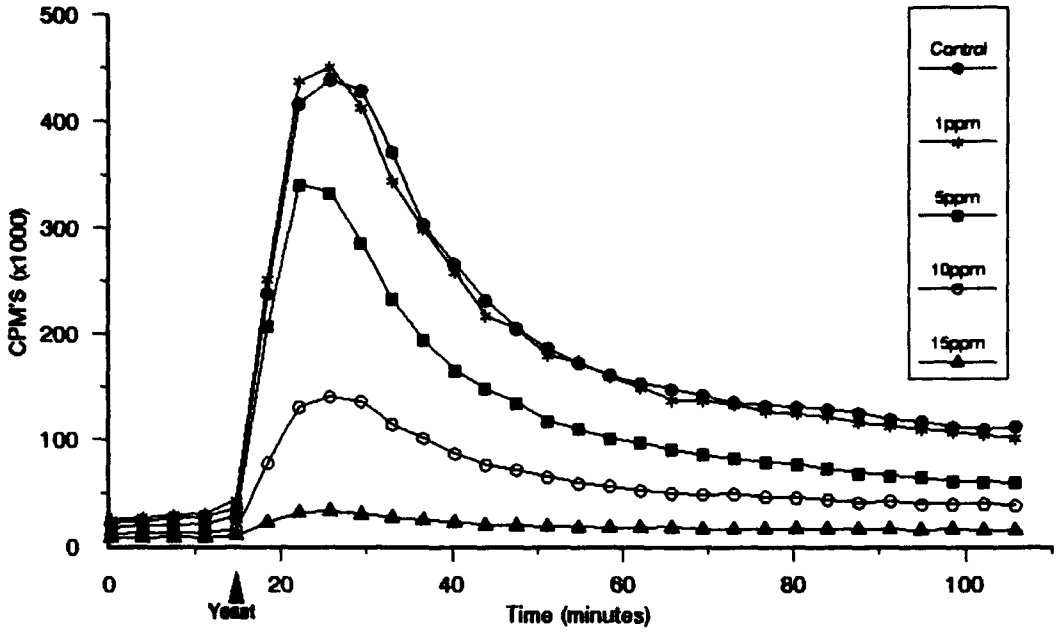


FIGURE 2

Effect of Twenty-Hour Exposure to Particulate Brass on Oyster Hemocyte CL.



The figures show representative data from aliquots of a single cell pool, Table 1 presents cumulative data derived from 5-6 cell pools for each exposure time. The dose dependent nature of brass dust inhibition of both resting and stimulated CL parameters is obvious, as is the increased intensity of the response when measured at 20 h, compared to 1 h. The data were used to estimate the brass concentration required for 50% inhibition of total CL activity (EC_{50}) and the concentration required to kill 50% of the cells (LD_{50}) were determined: 1 h EC_{50}) = 27 μg/ml, 1 h LD_{50} = 273 μg/ml; 20 h EC_{50} = 9 μg/ml; 20 h LD_{50} = 67 μg/ml. Clearly, these are sublethal effects that can be measured with sensitivity.

TABLE 1

Percent Inhibition of Hemocyte CL by Exposure to Particulate Brass.

	1 Hour Exposure				20 Hour Exposure			
	5	10	20	30 µg/ml	1	5	10	15 µg/ml
Bkg	18±21	45±6	72±6	88±9	15±24	53±24	73±13	83±11
Peak CL	9±10	27±11	56±12	73±9	18±15	49±30	76±18	91±10
Total CL	11±8	42±4	66±5	76±8	14±9	51±25	76±17	91±9

The brass dust used in this study was composed of 73% copper, 26.5% zinc and 0.5% aluminum. The hemocytes were exposed to the dust via particles and metal ions (and/or complexes) in the medium, as well as via endocytosis and subsequent breakdown of the particles. Preliminary work showed that brass leachates (400 ppm in estuarine water,, all particles removed after 1 wk extraction) also could cause CL inhibition in oyster hemocytes. The leachates contained copper and zinc concentrations in excess of environmentally relevant levels. it was also shown that copper ions, from copper sulfate, produced CL inhibition after incubation with hemocytes.

CONCLUSIONS

Exposure to brass dust, or aqueous leachates of the dust, will mediate a dose-dependent inhibition of oyster hemocyte chemiluminescence (CL). Copper, the major constituent of this agent, was directly involved in this suppression. Since CL is correlated with cellular antimicrobial activity and hemocytes are the oyster's main line of defense against infectious diseases, metal-induced CL inhibition was interpreted as a measure of immunosuppression. Thus, it appears the CL is a useful method to identify potential environmental immunomodulators in an important aquatic organism. Similar CL findings are reported with equivalent mammalian cells exposed to sublethal concentrations of metals.[12] Therefore, we proposed that hemocyte CL studies can provide not only information on immunotoxicants for bivalves, but also the results have a good potential for interspecies extrapolation.

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Dr. Anderson received a B.S. from Drexel University (1961), a M.S. from Hahnemann Medical University (1968), and a Ph.D. from the University of Delaware (1971). After postdoctoral experience in the immunology laboratory of Dr. Robert A. Good at the University of Minnesota (1971-3), he worked at the Sloan-Kettering Institute for Cancer Research in New York, where he headed the Laboratory for the Study of the Phylogeny of Cancer and Immunity (1973-82). He served as Research Biologist/Immunologist at CRDEC at APG from 1982-86; currently, he is a Professor at the Center for Environmental and Estuarine Studies, University of Maryland. His publications on various aspects of comparative immunology and carcinogenesis include about 45 primary papers, 25 book immunotoxicological studies of marine invertebrates, fish and mammals; in addition, the involvement of blood cell-produced oxyradicals in response to infectious disease of oysters is under study. Editorial duties and other peer-review activities include serving on Editorial Boards (J. of Invertebrate Pathology and J. of Developmental and Comparative Immunology), founding co-editor of Reviews in Aquatic Sciences, serving on the NIH Tropical Medicine and Parasitology Study Section (AHR), and reviewing grant applications and manuscripts submitted to various agencies and journals. Current educational activities involve directing the research of graduate students in the University-wide Toxicology Program, participating in team-taught courses in aquatic toxicology, serving on various search committees, and serving on the Faculty Senate. He is also active in several professional societies: American Association of Immunologists, American Society of Zoologists (Program Officer '81-'83; Public Affairs Committee '91-'93), International Society of Developmental and Comparative Immunology, Society for Invertebrate Pathology (Secretary '89-'91, Trustee '91-'93), International Society of Developmental and Comparative Immunology, Society for Invertebrate Pathology (Secretary '89-'91, Trustee '91-'94), Society of Sigma Xi, and Society of Toxicology.

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EARTHWORMS AS SUBSTITUTES FOR RODENTS IN METAL TOXICITY

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ABSTRACT

Earthworms (*Lumbrisis terristris*) are being investigated in our laboratories as potential substitutes for rodents for obtaining preliminary information on the toxicity of metal ions. Although these are two disparate species, the data obtained from using earthworms as experimental subjects can be related to the information available in the literature on rodents, especially mice. Both species contain the "detoxification" enzymes, catalase and glutathione-S-transferase. Acute toxicity of some metals has already been determined, and the earthworm is less sensitive than the rodents to metal toxicity. The relative ratios of toxicity are parallel in both species. Administered intraperitoneally, selenite is more toxic than selenate by a relative ratio of 3:1; this is the same in earthworms and in mice. Nickel acetate is somewhat less toxic than either nickel chloride or sulfate; the ratios of toxicity of the acetate to either the sulfate or chloride is the same. After challenging mice with a cadmium compounds, two isoforms of metallothionein are induced; the earthworm acts identically. Catalase activity in the liver of rats is first inhibited by cadmium; later in time, the activity returns to normal, and later may exceed base line values; the earthworm catalase acts similarly. The substrate 4-nitoquinoline-N-oxide is not as effective as 1-chloro-2,4-dinitrobenzene for glutathione-S-transferase in rodents as well as in earthworms. It appears that earthworms can be employed to obtain relative toxicity information, especially for comparing the order of toxicity of two or more compounds. Earthworms are cheap, readily available and easy to maintain.

INTRODUCTION

Certain segments of the public are pressuring the scientific community to find alternatives to animals for toxicology research. As a result of this, many investigators are using quite different systems as substitutes for experimental animals [1,2]. Among these are cell cultures; however, these cell systems are limited; they do not mimic a metabolizing rodent, as an example. Other test species mentioned in the literature are *Drosophila melanogaster,*

nematodes[3], planaria [4], and even *Daphnia magna*[5]. Not all of these species will give reproducible results in toxicological testing.

For toxicological investigations of metals, we have required a species with a metabolizing system containing some of the "detoxification" enzymes which are found in the mouse liver. The common earthworm, *Lumbrisis terristris* does possess catalase[6] and gluthathione-S-transferase (GSH-S-t)[7]. It can be assumed that these worms also contain super oxide dismutase[8]. Thus, this worm was selected to study the acute toxicity of some metal compounds. Worms are cold-blooded animals, and their anatomy and physiology are well documented[9].

Our results on metal ion toxicity were compared with known values from rodents. Also, these worms were compared to mice for the actions of metal ions on enzymatic activity of catalase, and glutathione-S-transferase.

To date, the following investigations were carried out in our laboratory:

1. The acute toxicity of selenite vs. selenate;
2. The relative toxicity of three nickel salts, the acetate, chloride and sulfate;
3. The induction of metallothionein after a challenge with cadmium chloride;
4. The inhibition action of a variety of metals on the catalase activity of the earthworm;
5. A comparison of two substrates, 1-chloro-2, 4-dinitrobenzene (CDB) and 4-nitro -quinoline-N-oxide (4NQO) on the activity of GSH-S-t;
6. The effect of two dimercapto compounds as arsenic antidotes, *meso*-dimercaptosuccinic acid (DMSA), 2,3-dimercapto-1-propanesulfonic acid (DMSA). Comparisons were made with British Anti-Lewisite (BAL) on three arsenic compounds-- arsenate, arsenite and phenyl dichloroarsine (PDA).

BRIEF EXPERIMENTAL INFORMATION

Procedure for acute toxicity:

Worms: The worms were purchased from a local bait shop. They were kept in potting soil fortified with moist corn meal. The holding cages were kept in the cold room at 15°C. When used, the worms were sorted by length and weight. They were usually 3-5 cm. in length and weighed between 4-5 grams. The actual ages were not known.

Each worm was rinsed with distilled water, gently pressed to remove the soil, blotted dry and weighed in order to calculate the appropriate injection dosages. The worms were then placed on a plastic bag containing ice to minimize activity. The measured amount of saline solution, either as a vehicle control or containing a known concentration of the metal compound was injected into the coelom directly behind the cliteium at an acute angle to avoid

entering the alimentary tract. They were then placed in a beaker, and allowed to warm to room temperature. Mortality was noted at 2, 4, 8, 24 and 48 hours. Between 5-10 worms were tested at every dose level. Each level was repeated 3-5 times. Lethality vs. dose was plotted on probit paper. The LD_{50} was estimated from the graph.

Procedure for other tests:

For the determination of metallothionein (MT), the worms were first treated with a sublethal dose of cadmium chloride (with tracer amounts of ^{109}Cd), and after a time lapse, the worms were homogenized, and the supernatant separated on Sephacryl S-200 columns. The metallothionein was determined indirectly by analyzing the cadmium by atomic absorption spectroscopy and by liquid scintillation counting.

For the arsenic antidote studies, the worms were first injected with the specific arsenic compound to determine its relative LD_{50} graphically using probit paper. The experiment was repeated for each of the three antidotes, by injecting the arsenic compound and then the antidote. The new LD_{50} values were determined.

Catalase activity was determine either volumetically, by noting the rate of evolution of oxygen after hydrogen peroxide was added to a worm homogenate; or spectrophotometrically by measuring the rate of decomposition of the H_2O_2.

The substrates 1-chloro-2,4-dinitrobenzene and 4-nitroquinoline-N-oxide were tested spectrophotometrically for the activity of glutathione-S-transferase. The effectiveness of the two was compared.

The protective action of sodium selenate against acute toxicity of cadmium chloride was determined by pretreating the worms with 1% of the LD_{50} for the selenate and then injecting the cadmium salt.

SOME INTERESTING RESULTS
(some remarks and also comparison to rodent activity)

Acute toxicity of selenium compounds

The LD_{50} value of selenite and selenate in earthworms are 31 and 60 mg/Kg respectively; for the rat the values are 3 and 5. The rats are about 10 times more sensitive to the selenium compounds. However, the ratios of toxicity of selenate to selenite are similar in both species[10].

Acute toxicity of three nickel compounds

The acute toxicity of nickel acetate, chloride and sulfate were determined in the earthworms. The values (mg/Kg) are: 69, 52, 54. For the mice the values are, 32, 26, 21. The ratios of the toxicity of the chloride and sulfate to the acetate are similar in both species[11].

Antidotes to arsenic toxicity

The values of the acute toxicities of the arsenic compounds are given. These are followed by the values after the respective antidotes were injected.

Arsenite, alone=191.0; after DMPS=254.6; after DMSA = 222.3; and after BAL=211.9.
Arsenate, alone=519.41; after DMPS=841.0; after DMSA=607.6; and after BAL=531.2.
Dichlorophenyl phosphine (DCP), alone=189.5; after DMPS= 287.7; after DMSA=225.0; and after BAL=317.4.

BAL is more active against the organic arsenic compound. The dimercapto derivatives are effective against inorganic arsenic. The latter results are not as spectacular as those reported by Aposhian[12] in mice for inorganic arsenic compounds, however, the earthworms do show similar protection. DCP values were not found in the literature for rodents[13].

Induction of metallothionein

Following the injection of cadmium chloride and determining its acute toxicity[14], the induction of metallothionein was determined. Results indicated that two peaks were found by measuring the cadmium content of the supernatant by means of atomic absorption spectroscopy. The experiment was duplicated using ^{109}Cd. Similar to the action in rodents, cadmium induced two isoforms of metallothionein in the worms[15].

Effect of two substrates for GSH-S-t

CDB was superior to 4NQO as substrate for GSH-S-t. The 4NQO reacted with the earthworm cytosol non-catalytically which negated its effectiveness as a substrate[16].

Effect of heavy metals on catalase

The divalent ions of Cu, Zn, Fe, Pb, Cd and Ni at levels up to 1000ppm, mixed with worm extract had a moderate inhibitory effect on the catalase activity. Lead and copper were the most effective inhibitors. Either Cd or Ni when injected into the whole worm 48 hours before the catalase determination exhibited enzyme enhancement above the base line[17].

DISCUSSION

From the variety of experiments conducted with the earthworm (*Lumbrisis terristris*) it does appear that useful preliminary information can be obtained on the toxicity of metal

compounds. Although the worms are less sensitive than rodents to the acute toxicity of the metal compounds, the ratios of toxicity of one compound to another are almost the same in these two disparate species. The effect of metal ions on the enzymes of the worms are the same as that of the rodents.

Others have use worms for studying the toxicity of pesticides with similar results to rodents. For example, Karr, et al.[18] used an earthworm to evaluate d-limonene, a monocyclic monoterpenoid for toxicity and neurotoxic effects.

The European Economic Community (EEC) now recommends that earthworms be tested for the toxicity of chemicals, especially organic compounds[19]. They suggest that an artificial soil be used, and that the species, *Eisenia fetida* be the test subjects. Heimbach[20] compared the activities of both *Eisenia fetida* and *Lumbricus terristris* against a variety of toxic agents and found a correlation of 0.81; no inorganic agents were in their test compounds.

CONCLUSIONS

Worms are readily available, are cheap, and can be maintained easily. We can conclude, after a series of experiments on toxic aspects of inorganic ions, that this species shows promise for obtaining comparative information on the toxicity of inorganic compounds.

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FROG EMBRYO TERATOGENESIS ASSAY - XENOPUS (FETAX): A NON-MAMMALIAN METHOD FOR DEVELOPMENTAL TOXICITY ASSESSMENT

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ABSTRACT

FETAX is a 96-hour developmental toxicity test that utilizes the embryos of the South African clawed frog Xenopus laevis. It is useful in screening for the developmental toxicity of single chemicals or complex mixtures. It is approximately 90% accurate in identifying developmental toxicants. Research is in progress to identify suitable carrier solvents for hydrophobic test materials, to develop further an exogenous metabolizing system utilizing liver microsomes to allow testing of proteratogens, and to develop methods for testing environmental samples under field conditions. An ASTM standard guide for conducting the FETAX assay has been published. An Atlas of Abnormalities is available as a companion to the ASTM guide.

The Frog Embryo Teratogenesis Assay - Xenopus (FETAX) is a 96-hour, whole-embryo, non-mammalian developmental toxicity screening test that utilizes the embryos of the South African clawed frog Xenopus laevis. FETAX was first developed and standardized as a definitive assay for developmental toxicants by Dr. James Dumont and his co-workers[1] at the Oak Ridge National Laboratory in 1983. The assay originally was developed to assess the potential developmental toxicity of complex mixtures derived from the synthetic fuels program at Oak Ridge National Laboratory. The assay is based on a large body of information generated by a number of researchers in studies on normal embryonic development in which the developing Xenopus embryo was used as the model system. The assay, as it is currently performed, is useful in screening for the potential

developmental toxicity of both single chemicals, such as pharmaceuticals or commodity chemicals, and complex chemical mixtures, such as environmental samples.

Advantages/Disadvantages of Xenopus

There are a number of advantages in using Xenopus as a model system to study developmental toxicity. The adult frogs used for breeding can be raised in the laboratory and are available commercially. The adults are easy to maintain in the laboratory since they are very hardy and do not require live food. The most important advantage of Xenopus is that the adults can be induced to breed throughout the year by the injection of human chorionic gonadotropin to induce ovulation and mating. This is in contrast to native frog species which have defined breeding seasons. Adult Xenopus are very fecund. A normal mating of a single pair of adults can produce up to several thousand fertile eggs and thus easily can provide an adequate number of embryos for testing. One of the disadvantages of using Xenopus is that it is not a native species, so care must be taken not to release the adults, the fertile eggs, or the embryos to the environment. However, there is a positive aspect to this since the use of Xenopus eliminates the need to use native amphibian species which appear to be in decline.

Advantages/Disadvantages of FETAX

FETAX has a number of advantages as a screening test for developmental toxicants. It is a relatively inexpensive short-term assay. Embryos are exposed continuously to the test samples over a 96-hour period, from midblastula-early gastrula (stage 8-11) to stage 46.[2] At the end of this period, the assay is terminated and data are collected. The assay utilizes a whole embryo in which all of the developmental processes are intact. Since the period of exposure to test materials coincides with the period of primary organogenesis in the embryo, the impact of developmental toxicants on the processes occurring during this period can be assessed. The stage 46[2] larvae are transparent. This allows for the easy identification of malformations in internal organs such as the heart and the gut. The use of FETAX as a developmental toxicity screening test permits a reduction in the number of laboratory mammals required for classical teratogenesis assays. There are several disadvantages associated with FETAX. The assay is an aqueous-based system, which, while an advantage for the testing of aqueous-based environmental samples, such as groundwater or waste water, presents problems for the testing of hydrophobic materials. This incompatibility requires the use of carrier solvents, such as acetone, DMSO, or triethylene glycol[3,4,5,6] to allow the testing of these hydrophobic materials. The use of these solvents introduces the possibility of solvent-test material interactions[4,5] and thus the results of any assays in

which a carrier solvent is used should be viewed with caution. The developing embryos lack a metabolic activation system for xenobiotics. Because of this, an exogenous metabolic activation system must be included in the assay if proteratogens are to be tested in FETAX. Proteratogens are compounds that require metabolic activation to become active developmental toxicants. The addition of the metabolic activation system is especially important if FETAX is to be used to assess the potential developmental toxicity hazard of a test material for mammals or humans. Finally, an obvious disadvantage of FETAX is that the assay fails to duplicate the placental function or the maternal-embryo interactions found in mammals.

FETAX Uses

FETAX can be used as a screening test in ecotoxicology or in the identification of potential mammalian and human health hazards. In ecotoxicology applications, it can provide information on the developmental toxicity of individual chemicals or complex mixtures of chemicals that may impact amphibians and other species found in the environment. FETAX has been used to assess the potential developmental toxicity of complex chemical mixtures found in surface waters,[7] groundwater,[8] and sediments.[9] With the addition of an exogenous metabolic activation system,[3,10,11,12] FETAX can be used to evaluate the potential developmental toxicity of pure chemicals or complex chemical mixtures for mammals and humans. FETAX can be useful in toxicity reduction assays. In these assays, complex mixtures of chemicals are fractionated and each fraction is tested for developmental toxicity with FETAX. Once the developmentally toxic fraction is identified the components of that fraction can be identified and steps can be taken to reduce or eliminate the toxic components thus reducing the toxicity of the complex mixture. An example of this approach to toxicity reduction is the work of Dr. Mendel Friedman at the U.S. Department of Agriculture in Albany, CA.[13,14] Friedman is using genetic engineering methods in an effort to reduce or eliminate developmentally toxic alkaloids found in potatoes and tomatoes. FETAX is being used as a screening test to evaluate the success of this effort. Schultz and Dawson have been using FETAX as a short-term assay for developmental toxicity in an attempt to relate the molecular structure of chemicals to their toxic activity.[15,16,17] FETAX can be used to evaluate the effects of metabolism on the developmental toxicity of xenobiotics. Chemicals can be subjected to metabolism <u>in vitro</u> through the use of an exogenous metabolic activation system incorporated in FETAX or known metabolites of the chemicals can be tested directly in FETAX. Another use of FETAX is in the investigation of the cellular and molecular mechanisms of developmental toxicity.[18,19,20]

FETAX Toxicity Endpoints

The primary toxicity endpoints used in FETAX are embryotoxicity as indicated by the 96-hr LC_{50}, teratogenicity as indicated by the 96-hr EC_{50}(Malformation), and growth inhibition as indicated by the Minimum Concentration to Inhibit Growth (MCIG). The MCIG is the minimum concentration of test material that significantly inhibits growth as determined by measurement of head-tail length of the 96-hr larvae exposed to the test material. The 96-hr LC_{50} and the 96-hr EC_{50} are determined using probit analysis. The MCIG is determined by the t-Test for grouped observations at $p<0.05$. In addition, developmental retardation is assessed by the determination of the stage of development attained by the exposed embryos by the end of the 96-hr exposure period. The Teratogenic Index (TI) also is calculated [TI=96-hr LC_{50}/96-hr EC_{50}(Malformation)]. Additional toxicity endpoints which have been used occasionally include changes in normal locomotion or swimming behavior, abnormal pigmentation, and failure or delay in hatching into free-swimming larvae.

FETAX Test Methods

A detailed description of the procedures used in the performance of FETAX can be found in the American Society for Testing and Materials (ASTM) Standard Guide for Conducting the Frog Embryo Teratogenesis Assay - Xenopus (FETAX).[21] Sexually mature adult South African clawed frogs, Xenopus laevis, are obtained from a commercial breeder (Xenopus I, Ann Arbor, MI). The breeding stock is held in aquaria connected to a flow-through well-water system in order to maintain water cleanliness. It also is possible to use a static holding system in which the water in the aquaria is periodically replaced with dechlorinated tap water. Since the frogs are extremely sensitive to the toxic effects of chlorine, it is very important that all traces of chlorine are removed before the frogs are exposed to the tap water. The water temperature for the adult frogs must be maintained at $23^{\circ}\pm3^{\circ}C$. Adult frogs are fed beef liver supplemented with liquid multiple vitamins. Breeding pairs are selected from the breeding stock. The day before the start of the test, both the males and the females of two to five breeding pairs are injected subcutaneously with an appropriate amount of human chorionic gonadotropin to induce ovulation and mating. Each of the injected breeding pairs is placed into a separate mating aquarium containing aerated FETAX solution,[22] a balanced salt solution. Ovulation, mating, and egg deposition occur during the night. The following morning, the adult frogs are removed from the mating aquaria and returned to the breeding stock to be used again for mating after approximately 6-8 weeks. The eggs deposited in the bottom of the mating aquarium are removed and inspected immediately for fertility and general quality. Upon completion of this inspection, the jelly coat on the eggs is removed by treating the eggs with a solution of

L-cysteine prepared in FETAX solution. Removal of the jelly coat enhances the susceptibility of the embryos to developmental toxicants by removing this potentially protective layer. Removal also reduces the adhesiveness of the eggs thus facilitating their manipulation. After the jelly coat has been removed, the embryos are transferred to dishes containing FETAX solution and again inspected for damage or abnormal development. Normally developing embryos in midblastula to early gastrula (Stage 8 to Stage 11)[2] are selected for use in the test. These embryos are randomly assigned to control or treated groups. Embryos from only one breeding pair are used in each test. Embryos from more than one breeding pair are never pooled. In the standard FETAX renewal exposure regimen, the embryos are exposed to the test material or the controls for 96 hours. During this period, the solutions to which the embryos are exposed are renewed every 24 hours. The temperature is maintained at $24^{\circ}\pm2^{\circ}$ in a controlled-temperature environmental chamber. At each renewal, embryos are examined for mortality and dead embryos are removed from the exposure dishes. At the end of the 96-hr exposure period, a final mortality count is performed and the remaining live embryos are fixed, examined for malformations, and head-tail length measurements are made with a computerized image-analysis system. The collected data are used to determine the 96-hr LC_{50}, the 96-hr EC_{50}(Malformation), the MCIG, and the TI. In general, an initial range-finding test is recommended. The range-finding test should consist of a series of at least seven concentrations of the test material that differ by a factor of ten. This test should be adequate to delineate the concentration range needed to establish the 96-hr LC_{50} and the 96-hr EC_{50}(Malformation). Three definitive tests should be conducted on each test material. Each test should consist of at least five concentrations which should be adjusted to allow for the determination of the 96-hr LC_{50}, the 96-hr EC_{50}(Malformation), and the MCIG.

Special Applications Methods

Currently, methods are being developed (Finch, unpublished) to permit the use of FETAX in a flow-through exposure system in a mobile biomonitoring laboratory which is to be used for on-site environmental toxicity assessment. FETAX is performed according to the ASTM Standard Guide for FETAX,[21] but the embryos are placed on the bottom of a mesh-bottom beaker (Fig. 1). The mesh-bottom beaker is made by removing the glass bottom of the beaker with a glass-cutting saw and replacing it with a section of plastic mesh which is cemented to the beaker with silicone sealant. The embryos are exposed to the test material in the flow-through system by suspending the mesh-bottom beakers in aquaria through which different concentrations of the test material is flowing (Fig. 2). The different concentrations are produced through the use of a solenoid-controlled dilutor apparatus. The developing embryos on the bottom of the beakers are bathed in the test material as

it exchanges through the plastic mesh bottom. After 96 hours of exposure in the flow-through system, the embryos are removed from the mesh-bottom beakers and processed according to standard FETAX procedures. This application of FETAX can provide on-site information about the potential developmental toxicity of chemicals found in surface waters, groundwaters, or waste-water effluents.

Linder and co-workers[23] have been developing methods for *in situ* applications of FETAX at contaminated waste sites. They have developed a plastic mesh exposure cage (Figs. 3A and 3B) to allow *in situ* exposure of the developing Xenopus embryos. The exposure cage is placed into the test matrix (e.g., sediment or water column) at on-site locations and then secured with stainless steel stakes or other restraints. This *in situ* exposure method recently has been used at an abandoned mine site in Montana.[23]

Representative FETAX Results

One of the primary FETAX toxicity endpoints is the presence of morphological malformations in embryos exposed to developmentally toxic materials. These malformations can vary from single minor abnormalities to multiple severe malformations. Figure 4 illustrates the severe malformations produced in a stage 46[2] embryo exposed to 0.05 mg/l t-retinoic acid for 96 hours in FETAX. The neural tube has failed to fuse during neurulation. Consequently, this embryo has no brain or eyes but was alive at the conclusion of the test.

Typical FETAX results for a non-teratogen (saccharin), a weak to moderate teratogen (caffeine), and a strong teratogen (5-fluorouracil) are shown in Figs. 5 and 6. Figure 5 presents concentration-response curves for mortality and malformation, as well as the TI, for each of these compounds. The TI values represent the degree of separation between the malformation and mortality curves. The greater the TI value, the greater the potential developmental toxicity hazard of the compound. For example, there is a large separation between the malformation and mortality curves for 5-fluorouracil indicating teratogenic effects are observed at far lower concentrations than embryo lethality. At concentrations where most or all of the surviving embryos are malformed there is little if any mortality. The probability of an embryo being alive and malformed at these concentrations is very high. By comparison, the malformation and mortality curves for saccharin cover the same concentrations and cross-over. This gives saccharin a TI equal to 1. This indicates that the probability of an embryo being alive and malformed is equal to or less than the probability of being dead. Generally, in FETAX, TI values of <1.5 indicate low teratogenic hazard,[3,10,18,22,24] however, the degree of teratogenic hazard indicated by the TI can not be used alone to determine if a compound is a teratogen or not. Courchesne and

Bantle[18] tested actinomycin D on Xenopus embryos and observed a cross-over of the malformation and mortality dose-response curves similar to that noted for saccharin (See Fig. 5). The calculated TI for actinomycin D would have been <1, indicating a low teratogenic hazard. However, the malformations induced in embryos exposed to this compound were so severe that it was considered teratogenic.[18] Growth inhibition appears to be a very sensitive toxicity endpoint that can be used to aid in the determination of the degree of teratogenicity of a compound. Figure 6 relates the concentration of the test compound as a percent of its 96-hr LC_{50} to its inhibitory effect on growth as indicated by the percent of mean control length attained by the exposed embryos. The MCIG for saccharin, caffeine, and 5-fluorouracil were 92.5%, 38.9%, and 7.4%, respectively, of their respective 96-hr LC_{50} values. As the teratogenicity of the compound increases the rate of growth inhibition and the total reduction in length increase proportionally. Dawson et al.[24] suggest that compounds with significant teratogenic potential generally inhibit growth at concentrations <30% of the respective 96-hr LC_{50} values.

By adding an exogenous metabolic activation system based on purified rat-liver microsomes to FETAX it is possible to use FETAX as a test to screen for potential mammalian and human proteratogens.[3,10,11,12] Figure 7 illustrates the effect of the incorporation of a metabolic activation system in FETAX in which the proteratogen cyclophosphamide was tested. Metabolic activation shifted the both the dose-response curves for malformation and mortality to the left, i.e., to lower effective concentrations. In addition, activation increased the distance between the two dose-response curves which resulted in a greater TI (TI=3.5 vs. 1.3). In effect, metabolic activation increased the developmental toxicity of cyclophosphamide.

Current Status of FETAX

Currently, the U.S. Army Biomedical Research and Development Laboratory at Fort Detrick is sponsoring, under contract to Dr. John Bantle of Oklahoma State University, an extensive interlaboratory validation study of FETAX. This study involves the participation of six independent laboratories. The purpose of the study is to determine the accuracy and reproducibility of the results of FETAX tests performed on coded samples of known mammalian teratogens and non-teratogens. The preliminary results of this study indicate that FETAX is very accurate in distinguishing between teratogens and non-teratogens and that the results for a given test compound are very reproducible among the participating laboratories. Approximately 100 compounds have been tested in FETAX in earlier intralaboratory validation studies. The results of some of these tests are summarized in Table 1. In these studies, it was found that the overall predictive accuracy of FETAX for the identification of mammalian teratogens and non-teratogens was

approximately 90% when the test incorporated an exogenous metabolic activation system. Intralaboratory validation studies of FETAX with new test compounds are continuing.[25] Studies to identify appropriate carrier solvents for use with hydrophobic test materials in FETAX, as well as their potential interactions with the test materials, are in progress.[4,5] Further development of the exogenous metabolic activation system is continuing.[12] In order to facilitate FETAX's further validation and establishment in new laboratories, an American Society for Testing and Materials (ASTM) standard guide for FETAX has been published.[21] The Atlas of Abnormalities: A Guide for the Performance of FETAX[26] also has been published. This atlas is intended as a companion to the ASTM standard guide for FETAX. The information and illustrations contained in the atlas provide a basis for the initial establishment of FETAX in a laboratory, as well as act as a guide in the identification and interpretation of developmental abnormalities observed in embryos during the performance of the test. Copies of the ASTM standard guide for FETAX, as well as the Atlas of Abnormalities are available free from Dr. Robert A. Finch or Dr. John A. Bantle.

CONCLUSIONS

The increasing costs of performing mammalian-based developmental toxicity tests, as well as the desire to reduce the number of laboratory mammals utilized in these tests has stimulated the development and validation of short-term non-mammalian screening tests to assess the developmental toxicity of a wide variety of pure chemicals and complex mixtures. The Frog Embryo Teratogenesis Assay - Xenopus (FETAX) provides a rapid, low cost alternative to the mammalian developmental toxicity tests currently in use. When combined with an exogenous metabolic activation system, FETAX is approximately 90% accurate in distinguishing between mammalian teratogens and non-teratogens. In addition, the results obtained with FETAX are very reproducible both on an intra- and interlaboratory basis. Final validation of FETAX will allow it to be used to screen and rank compounds for further developmental toxicity testing, to assess complex environmental mixtures for human and ecological effects, and as a tool for studying the basic mechanisms of teratogenesis.

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Figure 1. Mesh-bottom beakers used to expose developing Xenopus embryos to test material in FETAX performed in a flow-through exposure system.

Figure 2. A mesh-bottom beaker suspended in an aquarium through which test material is flowing.

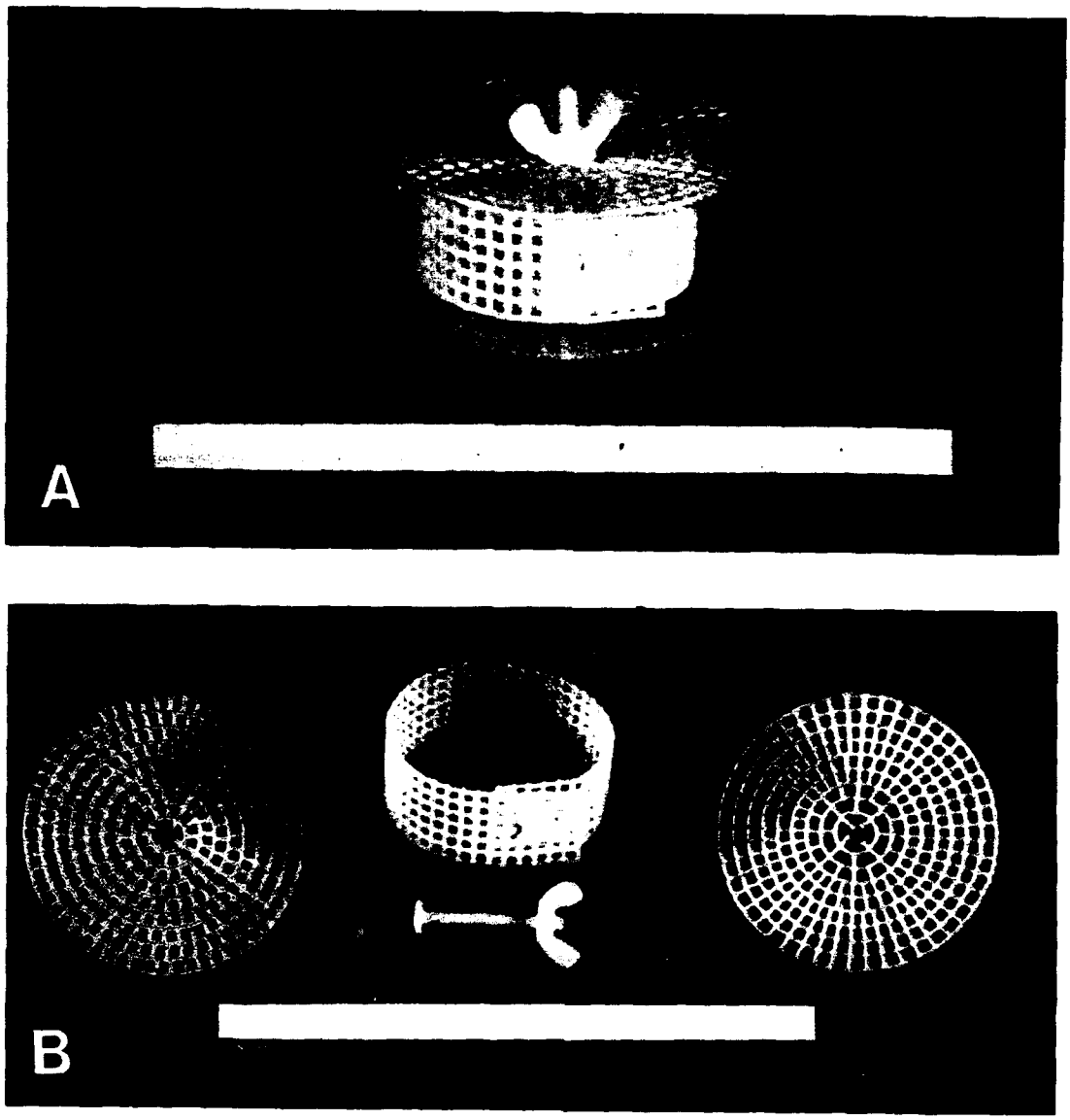

Figure 3. A, An assembled plastic mesh exposure cage used in <u>in situ</u> applications of FETAX. B, Component parts of the plastic mesh exposure cage.

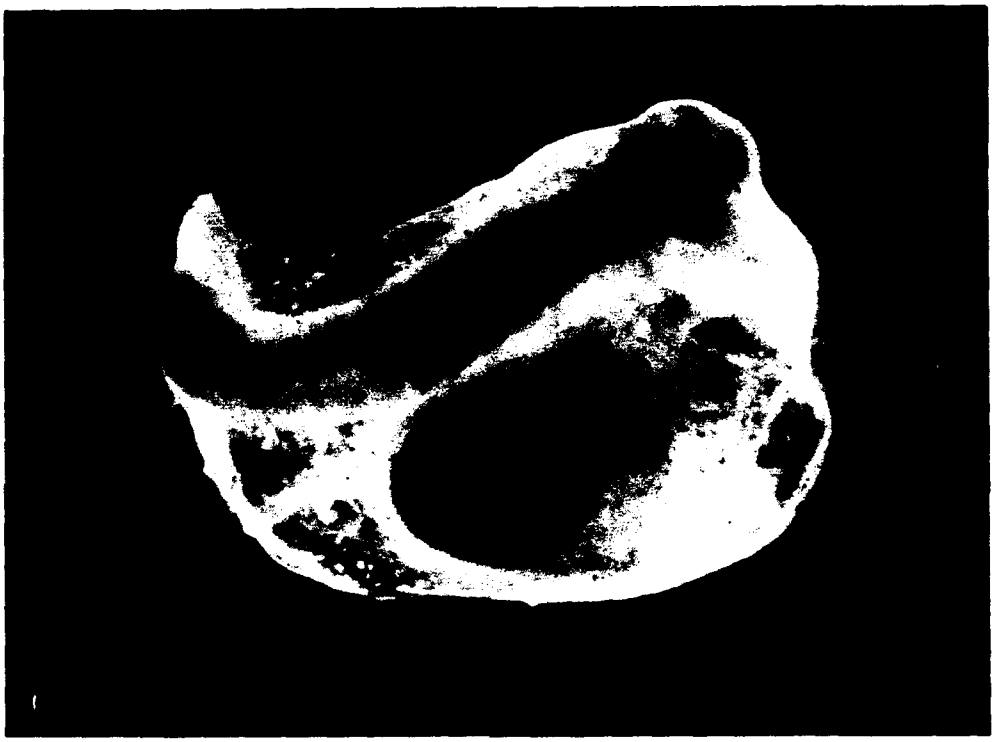

Figure 4. An example of the severe malformations produced in a stage 46 embryo which had been exposed to 0.05 mg/l t-retinoic acid during the 96-hr exposure period of FETAX. The neural tube has not fused and the embryo has no brain or eyes, however the embryo was alive at the conclusion of the test. All other organs are malformed as well. (Modified after Bantle et al.[26])

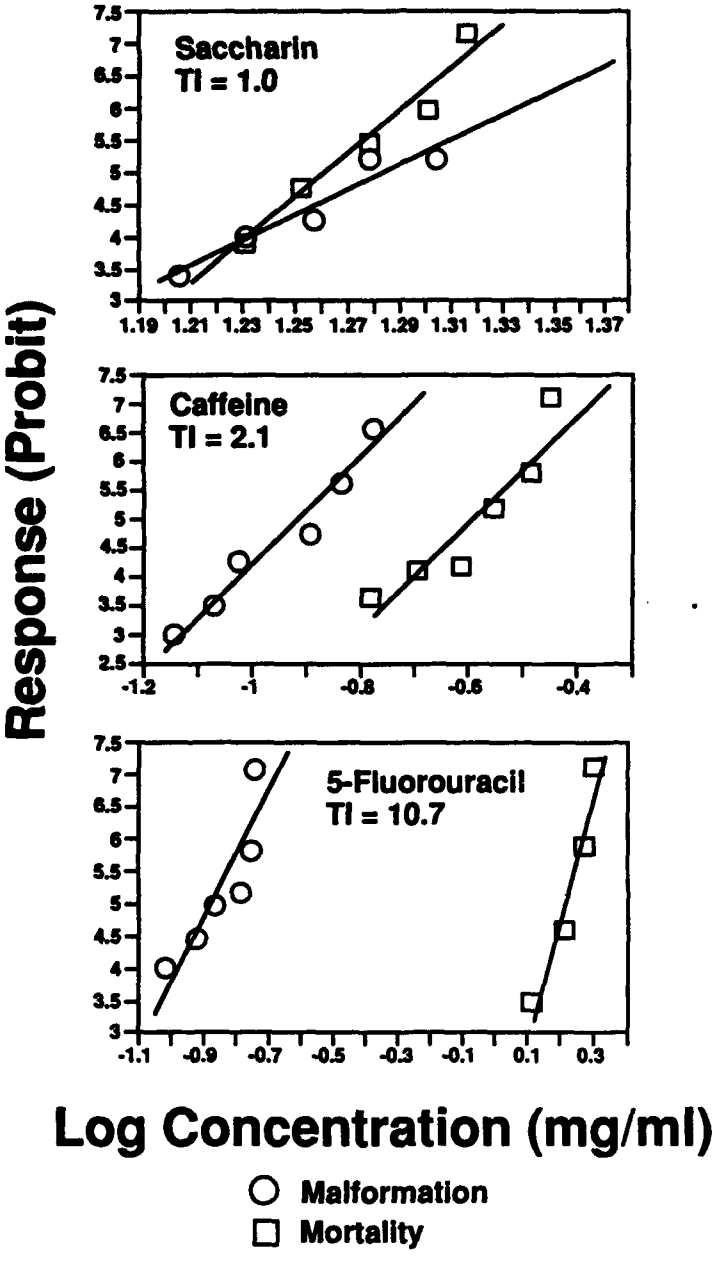

Figure 5. Concentration-response curves for mortality and malformation for Xenopus embryos exposed to saccharin, caffeine, and 5-fluorouracil. (Modified after Dawson and Bantle[22])

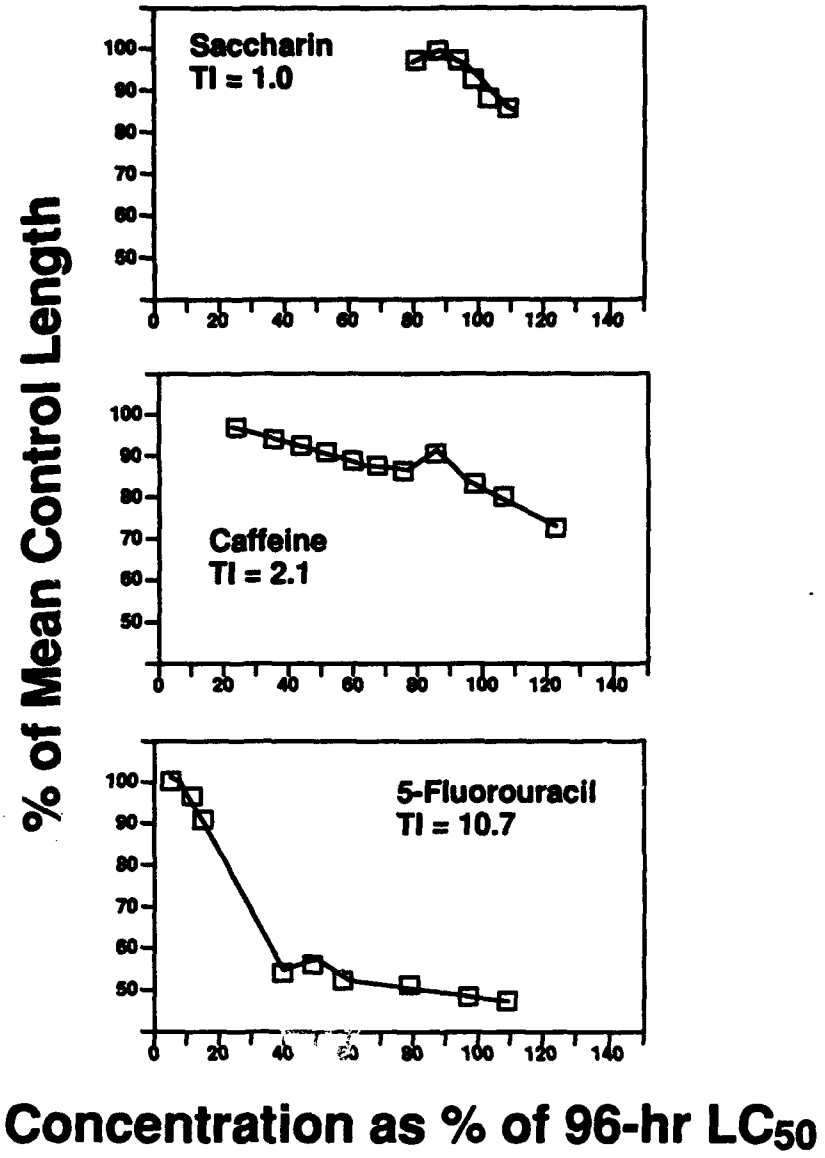

Figure 6. Representative growth curves for Xenopus embryos after 96 hours of exposure to saccharin, caffeine, and 5-fluorouracil. (Modified after Dawson and Bantle[22])

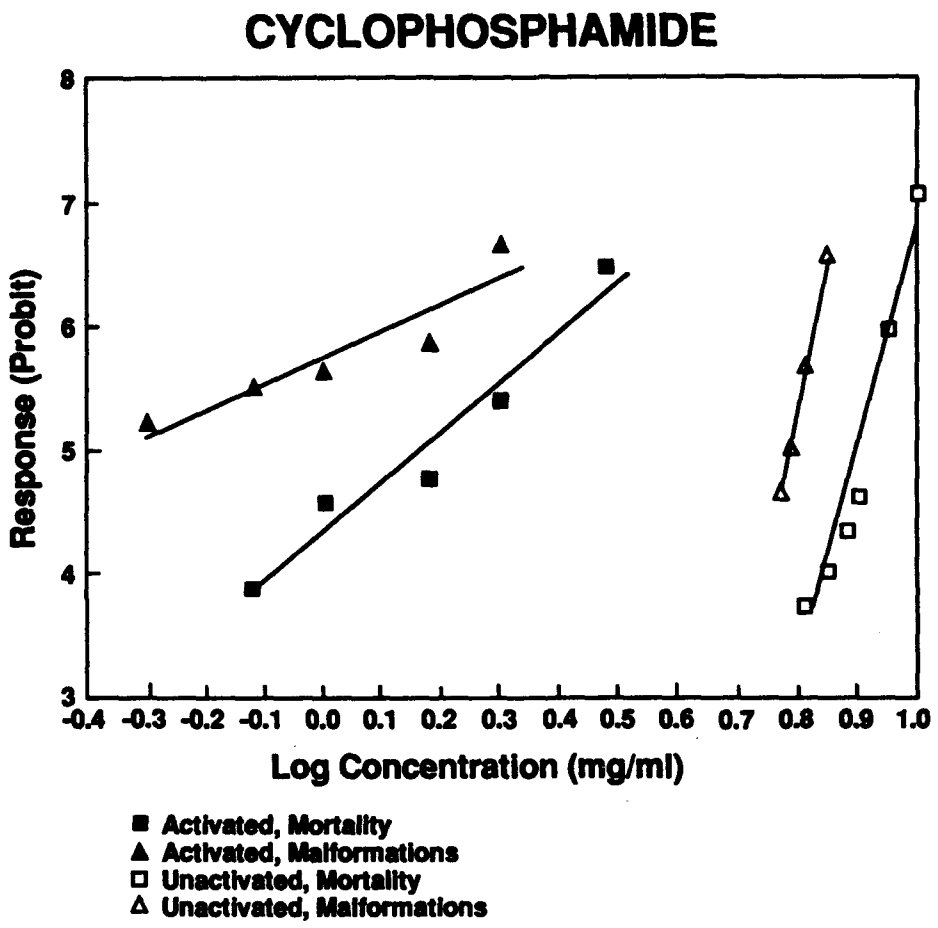

Figure 7. Concentration-response curves for mortality and malformation for Xenopus embryos exposed to the proteratogen cyclophosphamide with or without the incorporation of a metabolic activation system in FETAX.

TABLE 1

VALIDATION OF FETAX*

COMPOUND	TERATOGEN (TI>1.5)	NONTERATOGEN (TI<1.5)
DIRECT ACTING		
ACETAMINOPHEN		1.1
ACTINOMYCIN D		0.9
AMARANTH		1.0
5-AMINONICOTINAMIDE	560.0	
ASPARTAME		NT**
5-AZACYTIDINE	42.0	
CAFFEINE		2.1
COTININE		6.0
CYCLOHEXIMIDE		1.3
CYTOSINE ARABINOSIDE	7.1	
DIPHENHYDRAMINE	10.3	
ETHANOL		1.6
5-FLUOROURACIL	10.7	
HYDROXYUREA		4.7
ISONIAZID	37.9	
NAPHTHALENE		1.1
NITRILOACETATE		1.1
PENICILLIN G		1.1
PROPYLENE GLYCOL		1.2
d-PSEUDOPHEDRINE		1.4
PUROMYCIN		1.0
t-RETINOIC ACID	10.5	
SACCHARIN		1.0
SODIUM CYCLAMATE		1.1
TRITON X-100		1.1
URETHANE	3.3	
METHOTREXATE	23.0	

* The TI is the leading indicator of teratogenic hazard. However, the severity of the malformations and the MCIG relative to the 96-hr LC_{50} also must be considered in assessing the hazard.

** NT=Non-Toxic. An LC_{50} could not be determined and therefore a TI could not be calculated.

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THE USE OF SMALL FISH IN ENVIRONMENTAL CANCER SCREENING ASSAYS

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The use of fish species for screening for possible carcinogens has been the subject of increased interest in recent years. The need for establishing the hazard of contaminated military sites has also received increased scrutiny. The use of selected fish species for the purpose of conducting environmental hazard assessment will be discussed in this presentation. A specific study of trichloroethylene contaminated groundwater has been accomplished and a possible tumor promotion effect was noted at microgram per liter levels of contamination. The use of these species for environmental surveillance in the Army's Installation Restoration program will also be discussed.

Disclaimer

The views, opinions, and/or findings contained in this report are those of the author(s) and should not be construed as official Department of the Army position, policy, or decision, unless so designated by other official documentation.

Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NIH publication 86-23, 1985 edition.

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THE AQUATIC TOXICOLOGY OF ISOPROPYLAMINE, COMPARISON OF EXPERIMENTALLY DERIVED VALUES WITH STRUCTURE ACTIVITY PREDICTIONS

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Abstract

In order to assess the effects of isopropylamine (IPA), a potential industrial pollutant, on representative freshwater aquatic species, the aquatic toxicology of IPA was examined by conducting pH adjusted and unadjusted 48 hr acute (*Daphnia magna*), and 96 hr growth inhibition (*Selenastrum capricornutum*) toxicity tests. The pH unadjusted EC_{50} of IPA to *D. magna* was determined to be 91.6 mg/liter, and the EC_{50} when pH was adjusted to 7.0 was 197.6 mg/liter. A control toxicity test in which IPA was absent and pH was adjusted from 8.3 to 11.0 provided data relating the contributing role of high pH to toxicity. The lowest IPA concentration tested of 62.5 mg/liter inhibited algal growth by 92.4%, prohibiting EC_{50} calculation. The pH adjusted EC_{50} for *S. capricornutum* was 118.4 mg/liter. TOPKAT, a quantitative structure activity relationship (QSAR) program, predicted an EC_{50} range of 12.2 - 110. mg/liter using a *D. magna* model. An alternate calculation using rat oral LD_{50} data gave an estimated EC_{50} range of 16.5 - 110. mg/liter.

Introduction

Research that derives quantitative structure activity relationships has produced a variety of equations with predictive capabilities. Properly applied and validated, these equations should have the capability to enable the scientist to make better decisions as to environmental toxicity. Potential applications include participation in the risk assessments of contaminated sites containing multiple toxicants with limited datasets, and in the exploration of residual toxicity associated with the breakdown of a potentially new product. Unfortunately, it is usually difficult to examine the power of the model or equation with an unknown. As one of the goals of this paper, the prediction of two environmental QSARs are compared to experimentally derived results.

As part of a continuing series of environmental toxicity and hazard assessment studies, the toxicity of isopropylamine (IPA) to *Daphnia magna* and *Selenastrum capricornutum* was examined. In an effort to investigate the efficacy of alternate screening and range-finding methods for the *D. magna* toxicity tests, quantitative structure activity relationship (QSAR)

equations that are part of the program, TOPKAT, were used to generate model-specific toxicity estimates, which were compared to results of toxicity tests.

Isopropylamine, also known as 2-propamine and 2-aminopropane, is a colorless, flammable liquid with a strong ammonia-like odor. This strong base is miscible with water, alcohol, and ether, and is currently used in the synthesis of pharmaceuticals, pesticides, rubber accelerators, dyes and surface active agents, which may serve as potential sources of exposure. The chemical may enter the body via inhalation, ingestion, skin absorption, and eye and skin contact. Harmful effects and symptoms include irritation of the eyes, nose, throat and skin, pulmonary edema, visual disturbance, skin and eye burns, and dermatitis (1,2).

TOPKAT, developed by Health Designs, Inc. (HDi) (3), is an integrated program developed for the estimation of toxic effects of chemical structures using statistical QSAR techniques, such as multivariate regression and discriminate analysis. A number of structure-activity models have been developed by HDi for various toxic endpoints, including equations for a *D. magna* EC_{50} and a Rat Oral LD_{50} to *D. magna* EC_{50} for which the derivations have previously been described (4,5). Although log P (log of octanol/water partition coefficient) is the parameter most often used for QSAR studies of aquatic species, the models used in this study were constructed using molecular connectivity indices (MCI-shape descriptors), substructural keys, and biological endpoints (second model) as the parameters descriptive of the compounds in the models' database which contribute most to the explanation of the EC_{50}'s. For example, the following are six examples of the seventeen parameters used for the *D. magna* EC_{50} model: primary amine bound to an aromatic ring atom, aliphatic alcohol, oxygen-substituted aryl ester, benzene, valence path MCI, order 2, valence path-cluster, order 4.

Initial performance tests which validate the models' regression equation used the cross-validation method. Briefly, one chemical is removed from the data set, the equation is recalculated, and the omitted compound is estimated with the equation. The process is repeated for all compounds and the statistics calculated on the estimates. These results can be expressed as the fraction of compounds predicted within certain factors. The *Daphnia magna* EC_{50} model predicted 85.6% of the compounds from the data set within a factor of ten, however, the TOPKAT program includes specifications for the validation of an estimate. Users must incorporate toxicological experience and understanding of both the compound in question and those upon which the model was based, to provide confidence in the prediction. This communication addresses the aquatic toxicity of IPA reports the TOPKAT-generated *D. magna* toxicity estimates, and their validation.

Materials and Methods

Toxicity Tests: The 48 hr *D. magna* acute toxicity and 96 hr *S. capricornutum* growth inhibition tests conformed to applicable ASTM and U. S. EPA standards (6,7,8), and have been described previously (9.10). Daphnia cultures were originally obtained from Dr. Freida Taub at the University of Washington (Seattle, WA., USA) and were reared in the laboratory as described by Goulden et al (11) using hardened reconstituted water (7). Forty-eight hr tests were conducted using a 16:8 light/dark (315 ft. candles) cycle with temperatures maintained at 20 ±1° C. Due to increases in pH as percent of IPA in solution increased, two separate daphnid assays were conducted to determine the role of pH in IPA toxicity. The pH solution in the first assay ranged from 7.3 - 10.3. The second assay used a stock of IPA adjusted to pH 7.0 with 10% HCl, resulting in solution pH ranging from 7.1 to 7.4. The EC_{50}'s were calculated using the PROBIT analysis (12), and verified with graphically tabulated EC_{50}'s derived from mortality and concentration values (13). Additionally, a parallel set of controls was run in which no IPA was added, and pH was adjusted from pH 8.3 to 11.0 with 0.5 M NaOH. Percent mortality was plotted against pH in order to observe the effect of pH on the survivorship of daphnia. Water for this test and for

the culture of the *D. magna* used for the test, was obtained from a well source with treatment system that provides water at starting pH's of approximately 8.3. All other test and culture conditions remained the same as those above.

S. capricornutum was grown in a semiflow-through culture apparatus (14) on T82MV (15), and test conditions were the same as those for daphnia tests. Test vessels were inoculated with approximately 8.0 x 10^4 cells ml^{-1} of the algal stock. Two separate assays were again conducted to assess effect of pH on toxicity. Adjusted test pH was 7.0, while unadjusted test solutions ranged in pH from 7.5 to above 11.0 (pH probe not accurate above pH 11.0). Percent growth inhibition was calculated using the area under the growth curve (16), and plotted against concentration. Subsequent IC_{50}'s (concentration which inhibits 50% growth compared to control) were derived graphically from a least square regression line (17,18) and equation, calculated by using a function from the Macintosh compatible Cricket Graph scatterplot program.

TOPKAT QSAR Estimation and Validation: The structure of isopropylamine was entered into TOPKAT using the Simplified Molecular Input Line Entry System (SMILES) (Weininger, 1988). SMILES is a linear notation scheme for entry of chemical structures into computer databases. The language was developed to overcome complexities associated with the Wiswesser Line Notation method (20), and is much simpler and more user-friendly. The *Daphnia magna* EC_{50} and the Rat Oral LD_{50} to *D. magna* EC_{50} models were selected to obtain EC_{50} estimations (4). A two-step validation process was employed to ascertain the confidence for the toxicity estimates: (1) a determination was made as to whether the major substructures of the compound being estimated were present in the data base of the model selected, and (2) the data base was examined for structurally related compounds whose assay results and also TOPKAT-predicted estimates may either support or refute the estimate in question. This information, together with the user's own experience and knowledge of the compound, provides a measure of confidence that can be placed in the prediction. The resulting estimate was compared to the actual pH unadjusted EC_{50}.

Results

Results of assays appear in Table 1 and 2. The unadjusted 48 hr EC_{50} of IPA to daphnia is 91.6 mg/liter, while the pH adjusted EC_{50} is 197.6 mg/liter (as calculated by PROBIT). The parallel set of control run with no IPA indicated the fatal effects of increasing pH on *D. magna*. At pH 11.0, 35% of the daphnia population are immobile after 48 hr. The pH adjusted 96 hr algal growth toxicity test resulted in an EC_{50} of 118.4 mg/liter calculated by PROBIT). The unadjusted toxicity test would result in an EC_{50} below 62.5 mg/liter, the lowest concentration tested and which inhibited over 96% of the algal growth.

TABLE 1.
Summary of Daphnia Toxicity Tests

DAPHNIA MAGNA EC_{50} (MG/LITER)

	pH UNCONTROLLED	pH ADJUSTED
PROBIT	91.6; 95% confidence limits= 77.1-104.2	197.6; 95% confidence limits= 167.4 -230.6
GRAPHIC	89.4	195.3

TABLE 2.
Summary of Algal Toxicity Tests

SELENASTRUM CAPRICORNUTUM IC_{50} (MG/LITER)

	pH UNCONTROLLED	pH ADJUSTED
PROBIT	< 62.5	118.4; 95% confidence limits= 72.5-166.0
GRAPHIC	- - -	120.3

The TOPKAT *D. magna* EC_{50} model estimated the EC_{50} for unadjusted IPA to be 12.2 mg/liter. The characteristics that contributed to the estimate are presented in Table 3. A search for similar structures in the database used to compile the Daphnia QSAR revealed six compounds, 2-ethylhexylamine, allylamine, ethanolamine, ethylamine, n-butylamine, and cyclohexylamine that had a primary amine. In each case the *D. magna* EC_{50} QSAR overestimates the actual toxicity, just as it did with IPA. Comparisons of the actual EC_{50}s with the predicted values are also presented in Table 3.

TABLE 3.
Estimate of EC_{50} Using the TOPKAT *D. magna* 48 h EC_{50} Model

Name: Isopropylamine

Daphnia magna EC_{50} Model

Key		Cross product
Primary Amine (Noncyclic) R-NH2 (R=Alkyl)		0.961
Valence Adjusted Path MCI Order 1		0.437
Constant Term		2.287
	Total	3.685

Estimate of EC_{50} as Log(1000/Molar)=3.685 or 12.2 mg/liter

Compounds used in Validation of Estimate	Actual EC_{50}	Predicted EC_{50}
2-Ethylhexylamine CAS:104-75-6	2.2	4.44
Allylamine CAS: 107-11-9	110.0	14.1
Cyclohexylamine CAS:108-91-8	80.0	6.9
n-Butylamine CAS:109-73-9	75.0	30.8
Ethanolamine CAS:141-43-5	140.0	49.6
Ethylamine CAS:75-04-7	110.0	12.0

The TOPKAT Rat oral LD_{50} to *D. magna* EC_{50} predicted an EC_{50} of 16.5 mg/liter, similar to the estimate for toxicity predicted by the *D. magna* EC_{50} without the inclusion of rat toxicity data (TABLE 4). Characteristics that contributed to the estimate are included in TABLE 4. Five compounds were found in the database that were similar enough in structure to IPA for comparison: 2-ethylhexylamine, allylamine, n-butylamine, ethanolamine and ethylamine. The model overestimated the toxicity of all the compounds except ethanolamine.

TABLE 4.
Estimate of EC_{50} Using the TOPKAT Rat Oral LD_{50} to *Daphnia magna* EC_{50}

Key		Cross product
Rat Oral LD_{50}		0.808
Primary or Secondary Aliphatic Amine		0.868
Valence Adjusted Path MCI Order 2		0.077
Constant Term		1.802
	Total	3.555

Estimate of EC_{50} as Log(1000/Molar)=3.555 or 16.5 mg/liter

Compounds used in Validation of Estimate	Actual EC_{50}	Predicted EC_{50}
2-Ethylhexylamine CAS:104-75-6	2.2	1.8
Allylamine CAS: 107-11-9	110.0	13.3
n-Butylamine CAS:109-73-9	75.0	8.6
Ethanolamine CAS:141-43-5	140.0	213.3
Ethylamine CAS:75-04-7	110.0	25.5

Discussion/CONCLUSIONS

It is apparent from the reductions of toxicity in the pH adjusted tests, and the decrease in survivorship of daphnia at higher pH's, that pH contributes to the overall toxicity of IPA to both *D. magna* and *S. capricornutum*. Yet, pH is not the sole cause of immobility/mortality and growth inhibition. The impact of IPA on an aquatic ecosystem into which it has been introduced will depend, in part, on the system's buffering capacity with the addition of a base.

Upon running the TOPKAT program, it was found that the *D. magna* EC_{50} model overestimates the toxicity of IPA. Following the two-step validation process, the question of full representation of IPA in the model's database was satisfied. No major substructural features were outside of the descriptor-design set on which the equation was based, which can lead to a poor TOPKAT performance. To address the second step of the validation, the model's database was searched, and several very similar compounds were found whose actual assay results, and TOPKAT estimates, refuted the estimate generated for IPA. The daphnia model overestimates the toxicity of these similar compounds also, however an estimate for IPA can be considered valid by reporting a range of EC_{50}'s. This range, 12.2-110.0. mg/liter, is bracketed by the estimate, which is the lowest EC_{50} value, and the highest assay-derived EC_{50} available from the most similar data base compound. In reporting a range of EC_{50}'s,

confidence in this estimate is considered moderate. The fact that the actual EC_{50} for IPA falls within this reported range confirms the utility of the predicted results.

A similar performance was observed in the estimation of daphnid toxicity by the Rat Oral LD_{50} to *D. magna* EC_{50} model. The inclusion of the rat toxicity data apparently improves the performance significantly when the basic models are compared for predictive performance using a larger dataset (4). Moderate confidence in this estimate is also a result after validation by reporting a range of of EC_{50}'s from 16.5 - 110. mg/L, with the lowest value being the estimate, and the end value being the highest assay-derived EC_{50} available from the most similar data base compound. Once again, the actual EC_{50} for IPA falls within this reported range.

These results indicate that had TOPKAT been available to run prior to conducting the actual daphnia tests, it would have proven a valuable source for the determination of starting concentrations, thereby eliminating initial range-finding tests. TOPKAT also consistently overestimated the toxicity of the compound although the estimates were within an order of magnitude of the experimentally derived values. This consistent overestimation of toxicity may be a characteristic of the model in dealing with amine type compounds.

In the future it will be advantageous to continually test estimates and experimentally derived values in order to examine the ability of the environmental toxicity models included in TOPKAT. This could be done in a post hoc fashion as was done here or by consulting the literature for appropriate data. Verification of the utility of QSAR models using new data and compounds has the potential of providing estimates of hazard for situations not directly amenable to experimental manipulation.

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NANCY CHESTER

Ms. Nancy Chester works in the Environmental Toxicology Branch, Toxicology Division, at the Chemical Research Development, and Engineering Center (CRDEC), Aberdeen Proving Ground, MD. Ms. Chester graduated from Hood College, Frederick, MD in 1983 with a B.A. in biology and has been at CRDEC for about 8 years. She is responsible for conducting aquatic toxicology studies within the branch which include testing the toxicity of materials of interest to crustaceans, fish and other marine and freshwater organisms. This work serves to provide a dat base for CRDEC that is used for elementary risk assessment of the materials in question and represents a toxicological screening process which precedes further in-depth studies.

Ms. Chester first became involved in alternatives to animal testing when she became part of the research team which used aquatic animals lower on the phylogenetic scale for initial toxicologic testing, as just described. However, during her participation in the development of a partial data base for use in the determination of structure-activity models as an alternative to mammalian screening and for predicting ecological toxicity for risk assessments, Ms. Chester was further introduced to animal alternatives and became acquainted with a method of computer-assisted toxicological prediction. This is the subject of her presentation.

Most recently, Ms. Chester's research has led her to work with the Microtox assay, an assay designed to quickly quantitate the relative toxicity of a small volume sample by using living luminescent marine bacteria. This particular aquatic bioassay is currently being assessed for both sensitivity to local compounds/mixtures of interest, and comparability of the assay to other presently used solid and liquid toxicity screening methods.

STRUCTURE-ACTIVITY RELATIONSHIPS AND THE VALIDATION OF IN VITRO TOXICOLOGY TESTS

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ABSTRACT

Individual *in vitro* tests are limited by their inability to model the spectrum of responses produced in a whole animal test. Chemical and biochemical tests, however, in conjunction with structure-activity relationships, could provide a means of selecting a battery of *in vitro* tests to provide potency data for multiple responses. The selection of chemicals in the validation process needs to be systematized using methods developed in the field of quantitative drug design with reference to the intended molecular structure domain of applicability and corresponding chemical properties and molecular mechanism of action. *In vitro* tests can be limited by low solubility or high volatility of the test substance and non-linear relationships in the correlation of *in vitro* and *in vivo* potency.

INTRODUCTION

Ideally, an *in vitro* toxicology test should be able to provide qualitative information regarding both the potency and type of biological response of the whole organism. Many tests under development are designed to predict a single potency endpoint, for which validation consists of a comparison of experimental potency in the whole animal study with respect to this single *in vitro* endpoint.

Toxicologists are trained not only to report defined endpoints in whole animal experiments, but also to make a variety of qualitative observations that can provide important clues to the ability of a chemical to elicit additional adverse effects. The inability of most *in vitro* tests to model the spectrum of effects observed in whole animal experiments represents a limitation in their use as animal replacements. A potential bridge between *in vitro* and *in vivo* tests could be the use of *decision tests* that provide guidance in selecting appropriate *in vitro* endpoints for testing. We can define *decision tests* as triggers for *in vitro* hazard tests, but not replacements. Such decision tests need not require biological cells or tissues, but could be based upon purely chemical and biochemical measurements, as for example, measuring a chemical's intrinsic reactivity or susceptibility to metabolic

transformation. Ideally, such tests would be able to categorize a chemical's mode and molecular mechanism of action. Knowledge of the molecular structure correlates for such mechanisms based upon existing data for similar substances is also of great value in this endeavor.

Mode and Molecular Mechanism of Action

Let us distinguish between the mode and molecular mechanism of action of a chemical. The former may be regarded as a pattern or cluster of common biological responses exhibited by a class of chemicals such as irritants, while the latter requires an understanding of the relationship between this group of biological responses and what actually takes place at the molecular level. Irritancy can arise by a number of different molecular mechanisms. Knowledge of the relationship between mode and molecular mechanism can provide the link for the selection of appropriate *in vitro* tests and their relationship to the corresponding whole animal tests [1].

Molecular Mechanism Domain

Molecular mechanisms (in ascending order of complexity) can be divided into the following general categories: narcosis (baseline toxicity), electrophile, proelectrophile, pharmacophore, and propharmacophore [1]. With increasing complexity, the number of parameters required to interpret the effect at the molecular level is expected to increase while the domain of applicability of an *in vitro* model is expected to decrease.

Narcosis Mechanism (Baseline Toxicity)

Narcosis represents the most fundamental toxicological mechanism and corresponds to baseline toxicity [2]. In the presence of more specific mechanisms, the narcosis response will be masked by more specific effects elicited at lower toxicant concentrations. Nonelectrolyte chemicals of low reactivity such as saturated aliphatic monohydric alcohols, ethers, amides, hydrocarbons, and chlorinated hydrocarbons are associated with a narcosis type response [3].

A number of validation test studies now exist within the *in vitro* literature based upon chemicals known to act by this mechanism. It has been known since the discoveries of Meyer [4] and Overton [5-6] at the turn of the century that the potency of such chemicals in a wide variety of whole animal and cellular systems are all correlated with their partition coefficients, which provide a means of calculating a minimum biophase active concentration. Since all of these data correlate with partition coefficient, they also correlate with one another. Validation studies based solely upon chemicals acting by narcosis can provide an unrealistic appraisal of the applicability of the test if it is proposed for use for chemicals acting by more specific mechanisms. In addition, chemicals acting by narcosis yield excellent correlations with *in vitro* data from virtually any such system and therefore provide very unrealistic assessments of the validity of such *in vitro* methods for other chemicals. This condition is similar to the problem of intercorrelation of properties related to such potencies, despite the fact that partition coefficient is the one of fundamental significance [7].

In fact, partition coefficient has been used in a number of quantitative structure-activity relationship (QSAR) studies. Thus, a baseline toxicity QSAR model,

$$\log(1/LD50) = 0.805\ X - 0.971 \log (0.0807\ 10^{X} + 1) + 0.924$$
$$(r=0.824;\ s=0.208)$$

for the rat oral LD50 (in moles/kg) has been derived from data on 54 saturated monohydric alcohols and monoketones, where X is calculated log P (n-octanol/water partition coefficient) [8]. A comparison of predicted and observed LD50 values for various chemicals was useful in categorizing their mechanism with respect to narcosis or more specific mechanisms [1].

Electrophile Mechanism

Chemicals capable of undergoing covalent bond formation with nucleophilic sites (e.g., sulfhydryl groups) within the organism such as benzyl chloride, allyl bromide, acrolein, chloroacetonitrile, ethylene oxide, α-nitrostyrene, and acetaldehyde [1] can act by an electrophile mechanism. The potency of such action is controlled by the intrinsic reactivity of such substances and their rates of uptake, transport, metabolism and excretion. The toxicity of electrophiles can be modeled by QSAR using a reactivity parameter (pseudo first-order reaction rate with a model nucleophile) and log P, respectively [9]. Electrophiles have been implicated in lachrymatory and vesicant effects [10], and p-nitrobenzyl halides have been used as model nucleophiles in assessing skin sensitization of electrophile toxicants [11].

An acceptable *in vitro* system for testing electrophiles would be able to simulate in the whole animal the effects of reactivity on the rate of covalent bond formation with toxicant target sites and loss via glutathione and other detoxicant nucleophiles, as well as the effect of lipophilicity on uptake, transport, and excretion.

Proelectrophile Mechanism

Proelectrophiles are chemicals requiring metabolic activation to serve as electrophile toxicants. In principle, at least one other parameter relating to this metabolic transformation (e.g., redox potential) would be required in a QSAR model. Examples of proelectrophile toxicants are propargyl alcohol (alcohol dehydrogenase activation), pentaerythritol trially ether (monooxygenase activation), and 1,3-dibromopropane (glutathione transferase activation) [1]. The toxicity to fish of nitroaromatic compounds has been related to a proelectrophile QSAR model [12],

$$\log(1/LC50) = 0.96 \log P - 8.81\ E_{½} - 0.68$$
$$(n=20;\ r=0.964;\ s=0.18)$$

where $E_{½}$ is the polarographic half-wave potential (a measure of rate of metabolic reduction to the electrophile toxicant).

Pharmacophore and Propharmacophore Mechanisms

Chemicals acting by pharmacophore and propharmacophore mechanisms (more common for drugs and agricultural chemicals than industrial chemicals) are able to undergo receptor mediated binding either with or without metabolic activation, respectively. Due to

the high degree of stereoelectronic specificity required for receptor binding. QSAR models for chemicals acting by these mechanisms are generally restricted to closely related structures. Useful molecular descriptors for such studies can be derived through simulated interactions with the putative receptor using molecular modeling methods [13]. Such chemicals would appear to require specialized *in vitro* tests, such as those involving receptor binding now employed in exploratory drug research.

Chemical Selection

The selection of chemicals for evaluation of *in vitro* tests seems to be an area that has been to a large extent neglected. In most studies, such selection seems to be done without benefit of a formal systematic procedure. As a result, such selection is made by (1) relying upon what chemicals of interest have already been tested with the corresponding whole animal test, (2) choosing readily available chemicals belonging to the class of interest, or (3) attempting to validate the test for all possible chemicals by choosing sample compounds from different categories of chemicals such as pesticides, industrial chemicals, metals, surfactants, etc. As used here, the term category refers to *ad hoc* classifications used for such studies, and should not be regarded as having any fundamental significance.

Basing such a choice upon what chemicals have already been tested in the whole animal may seem pragmatic, but may in fact provide a false sense of validity with respect to the larger universe of chemicals or even lead to an unduly pessimistic conclusion about the predictive value of the test with respect to a more limited special category of interest. Most members of the larger group may be useful for exploring only a small number of mechanisms, whereas chemicals belonging to a specific class of interest may all act by a common mechanism.

Application of SAR and QSAR to Chemical Selection

Structure-activity relationships (SAR) and quantitative structure-activity relationships (QSAR) can provide useful paradigms for systematizing the selection of such test chemicals [13]. Although the use of a finite set of chemicals for validation can never assure the predictability of a test for all chemicals, a thoughtful selection process can more efficiently achieve this objective and provide greater confidence in the applicability of the test.

The greatest confidence in such validation studies can be gained if the application of an *in vitro* test can be limited, at least initially, to a very well-defined, discrete class of chemicals. A major difficulty in choosing such a set of chemicals relates to the fact that the scope of such a category is in itself difficult to define. Within the QSAR paradigm, molecules are commonly compared in terms of their properties rather than in relation to the skeletal connections in the whole molecules. The use of such chemical parameters or molecular descriptors may seem like a simple solution to defining what chemicals should be tested, but a more difficult question arises regarding the choice and rationale for selecting properties and the extent to which these properties may contain redundant information. For example, partition coefficients between water and various immiscible organic solvents such as chloroform will be collinear with those of octanol/water in many, but not all chemicals. These partition coefficients, in fact, depend upon more fundamental properties including molar volume and the ability to serve as a hydrogen bonding donor and acceptor.

In the Hansch approach to QSAR, a hydrophobic (e.g., log P), electronic (e.g., pKa) and steric (measure of fit to site of action) property are used as parameters or molecular

descriptors in analysis as well as in chemical selection. For example, suppose an investigator were testing a series of para-substituted benzoic acid derivatives. Chemicals would need to be selected to represent the range of all three of these parameters likely to be encountered in future reliance upon the *in vitro* test. The chemicals should be chosen so that they are evenly distributed within this 3-dimensional space. There is an implicit assumption of lack of change in molecular mechanism of action within this group of chemicals.

Hansch has characterized the domain of such a testing space as "spanned substituent space" [14]. Compounds outside of this domain may be predicted, but such predictions should be regarded as extrapolations rather than interpolations.

An additional complication arises due to the inability to uniquely define the dimensionality of chemical space encompassed and the nature of these dimensions. This difficulty is related to the even more fundamental question of the relationship between the dimensionality of the test set and the larger universe of chemicals for which such predictions may be desired.

Water Solubility Related Limitations

Many *in vitro* tests consist of cells suspended in an aqueous medium to which the test substance is added. Potency is judged based upon the relative concentration required for a substance added to this phase to produce a defined biological response. Additional insight can be gained regarding the physicochemical limitations of such tests by their analogy to experience in testing chemicals using fish and other aquatic organisms in which uptake also occurs from a solution in water.

Testing substances with a reasonable degree of water solubility should not pose special experimental problems in an *in vitro* test, but this is not the case when testing highly insoluble compounds. In aquatic toxicology, it is not uncommon to artificially solubilize such compounds using a small amount of cosolvent that is miscible in water.

Nevertheless, it is essential to exclude the possibility of additional toxicity being contributed by such a cosolvent, and to insure that the cosolvent is used to produce more rapid dissolution and does not lead to testing above actual water solubility. Thus, for some substances, toxicity is intrinsically limited by water solubility, and the use of such supersaturated solutions may provide misleading results. Saturated solutions for highly insoluble substances can also be prepared efficiently with the use of generator columns. It is recommended that the actual test concentrations be determined analytically when testing such insoluble substances, and that the use of nominal concentrations be avoided as it has been the current practice in aquatic toxicology. Use of measured concentrations also avoids problems of loss due to adsorption to the glass walls of the test chamber.

For liquid solutes, there is a high correlation between water solubility and octanol/water partition coefficient [15]. However, for solutes that melt above room temperature, the correlation needs to be corrected for the transition from the solid to the liquid phase as part of the dissolution process [16], and chemicals with similar partition coefficients acting by the same mechanism would be expected to show equivalent cutoffs. Solubility decreases with increasing melting point for solutes having the same partition coefficient, and melting temperature is a function of intermolecular hydrogen bonding, symmetry and conformational flexibility. These factors should be considered in identifying such highly insoluble compounds and defining appropriate procedures prior to *in vitro* testing.

Problems from Testing Volatile Chemicals

Chemicals that are volatile from water require special experimental considerations to insure that the actual concentration is known and does not decrease with time. This can be accomplished, as in aquatic toxicology testing, by the use of a controlled closed system, continuous flow and analytical monitoring of the actual concentration over time. In designing studies for potentially volatile compounds, it is useful to obtain a prior estimate of volatility (air/water partition coefficient), which can be approximated directly from the chemical structure [17].

Nonlinear Relationships

According to Richardson's Rule, the toxicity of chemicals within a homologous series increases with increasing chainlength and molecular weight [18]. In the rat oral LD50 QSAR equation cited above for baseline narcosis toxicity, the form of the model gives rise to a nonlinear equation with maximum toxicity corresponding to a log P value of close to 2.

In fact, Richardson's Rule applies only if pseudo steady state thermodynamic equilibrium can be achieved between the site of administration and the site of action. This is not the case in whole animal studies except via gaseous or vapor administration. Richardson found such apparent relationships only because he did not investigate higher members of a series [18]. In aquatic toxicology and corresponding *in vitro* tests, linear relationships will be observed over a larger range of compounds within a series and the *in vitro* test could thus predict greater potency than would be observed in the whole animal test.

CONCLUSIONS

Chemical and biochemical tests along with structure-activity relationships could be used to select a battery of single endpoint *in vitro* tests to disclose the spectrum of biological responses observable in the whole animal test. QSAR models already exist for predicting the potency of some chemicals with respect to certain whole animal endpoints. In selecting chemicals for the validation of *in vitro* toxicology tests, careful appraisal needs to be made of the class or classes of chemicals for which the test is to be applied, along with corresponding molecular mechanisms of toxicity and domain of spanned substituent space parameters. Special procedures used in aquatic toxicology for testing very insoluble or volatile chemicals should be employed in corresponding *in vitro* tests. Nonlinear relationships are expected between potency measured in whole animal and *in vitro* tests due to differences in uptake, transport, metabolism, and excretion.

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Robert Lipnick received his Ph.D. degree in organic chemistry at Brandeis University in 1969 and was a postdoctoral fellow in the Chemistry Department at the University of Minnesota, and a Research Associate at the Sloan-Kettering Institute for Cancer Research in New York. He came to the EPA Office of Toxic Substances in 1979, and has been a Senior Chemist in the Environmental Effects Branch since 1985.

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In addition, he has presented numerous papers at national and international scientific meetings, including serving as a co-organizer for an EPA Symposium and chair of sessions at national and international meetings. He recently served as a member of the International Scientific Committee of the 4th International Symposium on QSAR in Environmental Toxicology. He is a member of the American Chemical Society, Society of Environmental Toxicology and Chemistry, QSAR Society, and the International Group for Correlation Analysis in Organic Chemistry.

Dr. Lipnick is interested in the correlation of chemical structure with physicochemical properties, spectroscopic properties, and chemical reactivity, and the correlation of chemical structure with toxicological mechanism of action and potency. He has applied these approaches at EPA in the assessment of the potential adverse effects of chemicals under conditions in which only limited toxicological test data were available.

Session VI: *In vitro* and Other Alternatives for Non-Carcinogenic Toxicological Endpoints

Co-Chairs: Drs. Eugene Olajos and June Bradlaw

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JUNE A. BRADLAW

Dr. June A. Bradlaw received her A.B. degree in Botany from Connecticut College, M.S. degree in Microbiology from the University of Maryland and Ph.D. degree (1974) in Microbiology from George Washington University. She is currently the Leader of the In Vitro Toxicology Team, Division of Toxicological Studies, Center for Food Safety and Applied Nutrition, Food and Drug Administration in Washington, D.C. and has been with FDA since 1965 as a member of the Genetic Toxicology Branch. Between 1981 and 1989, she also held the position of Adjunct Associate Professor of Microbiology at the George Washington University School of Medicine and taught a graduate level course on cell and tissue culture.

Dr. Bradlaw's research interests are centered about developing methods to improve the predictive value of cell, tissue, organ and embryo culture systems with respect to extrapolation to animals, and ultimately humans, for estimation of risks associated with chemical exposures. Her research focuses on *in vitro* toxicity testing approaches as either screening methods to detect chemical toxicants or to study cellular mechanisms associated with the toxicity of chemicals, nutrients or chemical-nutrient mixtures. Her research efforts involve primary rodent cell cultures of hepatocytes from perfused adult liver, endothelial cells from porcine vascular tissue, heart muscle cells and the quantitation of relevant endpoint measurements of toxicity at the cellular and subcellular levels of organization.

Dr. Bradlaw is currently the Secretary of the Tissue Culture Association and has Co-Chaired the Toxicity, Carcinogenesis, and Mutagenesis Evaluation (Cellular Toxicology) Committee. She represents FDA as a member of the Validation and Technology Transfer Sub-Committee for the Johns Hopkins University Center for Alternatives to Animal Testing, serves as a member of the Scientific Advisory Board, International Foundation for Ethical Research and both editor and reviewer for numerous cell toxicology journals. She is a member of Sigma Xi, The American Academy of Microbiology, American Society of Microbiology, American Society of Cell Biology and Graduate Women in Science.

TISSUE SLICES AS AN *IN VITRO* MODEL FOR STUDYING HEART, LIVER AND KIDNEY TOXICITY

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Tissue slices prepared from heart, liver and kidney were used to evaluate chemical toxicity in these target organs. A variety of chemicals were dosed onto these slices for 22 hours, after which tissue-specific toxicity was measured by vital dye uptake and histopathology. The results indicate that slices from different tissues discriminate among tissue-selective toxicants. Specifically, the cardiotoxicant doxorubicin was most potent in heart slices and the hepatotoxicant allyl alcohol was most toxic to liver. Results are also presented for rank-ordering the toxicity of chemically related metals and alcohols. This study demonstrates that an *in vitro* tissue slice battery can provide a useful tool for rapidly identifying target organ susceptibility, chemical potency and the mechanism of action of a wide variety of chemicals.

INTRODUCTION

Tissue slices have been used as an alternative *in vitro* method to primary cell culture. Historically slices were prepared with the Stadie-Riggs microtome or by hand, resulting in wide variations in thickness. These thickness variations cause thicker areas of the slice to become anoxic and necrotic following short periods (a few hours) of incubation; this problem confounded experimental results and limited the utility of slices as an *in vitro* model[1,2]. The development of the Krumdieck tissue slicer[3] allowed for the production of precision-cut tissue slices of uniform dimensions. Such slices are rapidly prepared under physiological conditions, are uniform in thickness and do not develop local regions of anoxic injury. Normal physiological processes are preserved in liver slices as measured by protein synthesis, intracellular potassium content, maintenance of ATP and cytochrome P-450 levels[4,5]. This slicing technique has also been used to evaluate chemical toxicity *in vitro* in kidney[6,7], lung[8] and heart tissues[9].

The methodology of precision-cut slicing can be easily adapted for preparing slices from the soft tissues of most species for *in vitro* investigations of chemical metabolism and toxicity. The specific goal of this study was to determine if slices prepared from different tissues respond differently *in vitro* to classic target organ-specific toxicants.

MATERIALS AND METHODS

Sprague-Dawley rats (Harlan) and New Zealand White rabbits (Hazleton) were used as a source of fresh tissue. Mercuric chloride, potassium dichromate, nickel chloride, cadmium chloride, zinc chloride, allyl alcohol, cis-platinum(II) diammine dichloride, trans-platinum(II) diammine dichloride, 3,4,5 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT) and tissue culture media were obtained from Sigma Chemical Company. Methanol was obtained from Mallinckrodt, ethanol from the Warner Graham Company, magnesium chloride from Aldrich Chemical Company and doxorubicin was purchased from Adria Laboratories. Serial dilutions of each of these test materials were made using tissue culture media as a diluent. Precision-cut tissue slices were made using a Krumdieck tissue slicer (K & F Research).

Animals were anesthetized with pentobarbital and their tissue rapidly harvested using sterile technique. Heart, liver and kidney were obtained from either rats or rabbits. Tissue and chemical comparisons were always made between tissues derived from the same species. Tissue cores and slices from each of these three organs were prepared using methods previously described[2,6,9]. Replicate slices were incubated in tissue culture plates in a 37 $^{o}C/CO_2$ incubator in tissue culture media in the presence of serial dilutions of the various chemicals for 22 hours. At the end of this time period slices were harvested for MTT and histopathological analyses.

Chemically-induced injury to slices was determined by measurement of MTT conversion as a marker of mitochondrial integrity according to the method of Mosmann[10]. Spectrophotometric analyses of converted MTT was performed with a UVMax microtiter plate spectrophotometer at an absorbance of 570 nm (Molecular Devices). For histopathological analyses slices were fixed, embedded in paraffin, sectioned with a microtome and then stained with hematoxylin and eosin.

RESULTS

The first phase of this study involved determining if the tissue slice models were suited for predicting the rank-order of toxicity of chemicals with well documented toxicity profiles *in vivo*. Cis- and trans-platinum were selected for study since the nephrotoxic potency of the cis isomer of is far more injurious than the trans isomer to the renal proximal tubule *in vivo*[11]. The results of this study using rabbit kidney slices dosed with serial dilutions of these chemicals (Figure 1) demonstrate the same rank-order of potency *in vitro* as observed *in vivo* for these stereoisomers.

A second experiment was conducted to determine if the tissue slice models were suited for predicting the rank-order of toxicity of another series of structurally related chemicals with well documented toxicity profiles *in vivo*. In this study the potency of three alcohols with significantly different *in vivo* hepatotoxic potencies were selected to evaluate the susceptibility of the *in vitro* liver slice model to alcohol-induced toxicity. The alcohols selected for this experiment included ethanol, methanol and allyl alcohol. Of these three compounds allyl alcohol is the most hepatotoxic *in vivo* by virtue of its hepatic alcohol dehydrogenase-mediated conversion to acrolein[12]. The results of this study using rabbit liver slices dosed with serial dilutions of these alcohols show that allyl alcohol is far more hepatotoxic than either methanol or ethanol *in vitro*, similar to the rank-order toxicity observed *in vivo* (Figure 2).

FIGURE 1

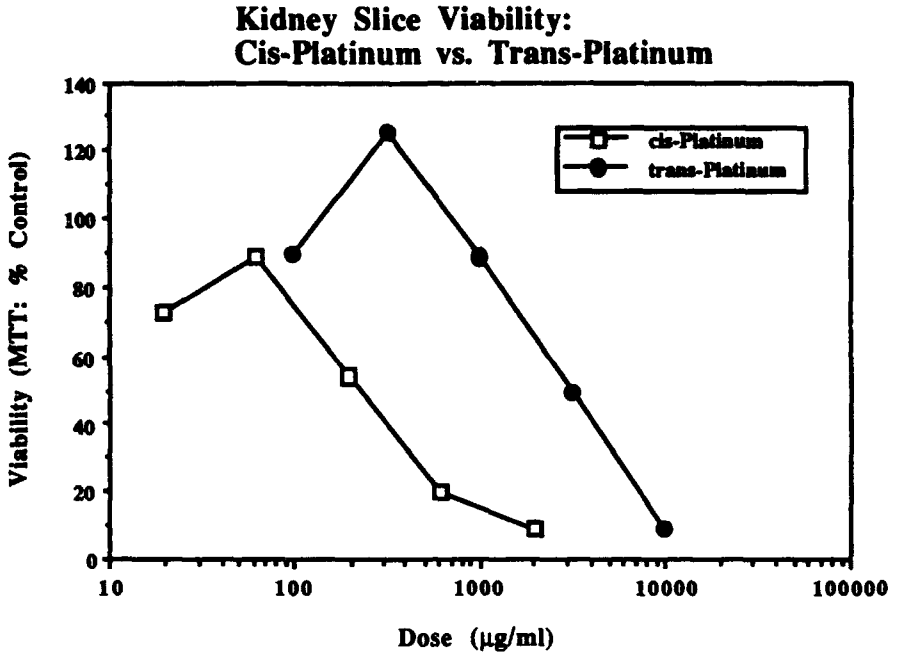
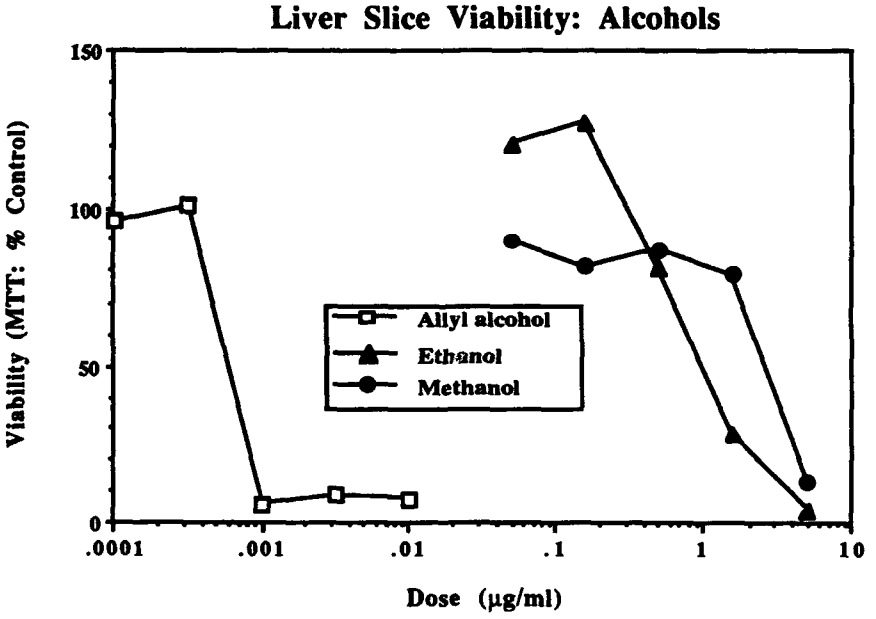
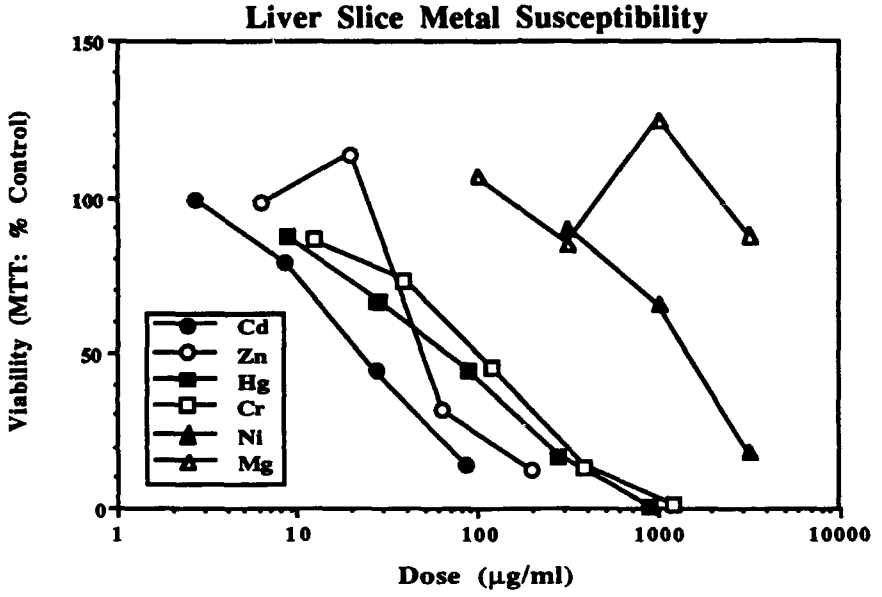


FIGURE 2



The rank-order of toxicity of a series of metals was evaluated to determine if the slice model could predict the toxicity of this group of related chemicals. In this experiment the potency of six different metal salts with significantly different *in vivo* hepatotoxic potencies were evaluated in the *in vitro* liver slice model. Three of the metals selected for this experiment are considered potent toxicants, including cadmium chloride, mercuric chloride and potassium dichromate. Zinc chloride, nickel chloride and magnesium sulfate were selected for comparison as metals with considerably less *in vivo* hepatotoxicity potential[13].

FIGURE 3



The results of this study using rabbit liver slices dosed with serial dilutions of these metals are presented in figure 3. Generally, the rank-order of potency for these metals *in vitro* correspond qualitatively to the potency of these chemicals observed *in vivo*, with the possible exception of zinc chloride (Figure 3). This discrepancy may be due to the poor absorption of this metal salt when administered orally *in vivo*. Thus, the enhanced toxicity of zinc chloride in the slice system is likely due to a higher concentration of this metal in contact with the target organ (liver) *in vitro* than is present *in vivo*. This hypothesis is supported by data comparing the acute toxicity of metals administered by different routes (Table 1). Note that the toxicity of mercury is not nearly so route-dependent as is zinc, suggesting that the systemic availability of zinc is very limited when administered orally.

TABLE 1

Mouse Acute Toxicity Data *In Vivo*[a]

Compound	Oral LD50 (mg/kg)	Intraperitoneal LD50 (mg/kg)
Zinc Chloride	350	31
Cadmium chloride	60	9
Mercuric chloride	6	6

[a]Data from Sigma Chemical Company

Although mercury and chromium follow similar dose-response curve of toxicity in this slice system, histopathological evaluations show that these metals can target different cell types in the kidney (14). Many chemicals have been demonstrated to target specific regions within tissue slices due to innate differences in cellular susceptibility[4,7].

A fundamental question in this study was to determine if the tissue slice models were suited for evaluating and differentiating among tissue-specific toxicants. Allyl alcohol was selected as a model hepatotoxicant. This compound is thought to selectively target the liver since it is converted to acrolein via the high activity of hepatic alcohol-dehydrogenase (ADH)[12]. By way of contrast the toxicity of allyl alcohol to kidney slices was also studied; renal ADH activity is approximately one-tenth of that found in liver[15], so that the conversion of allyl alcohol to the toxic intermediate acrolein is likely to be proportionately lower in the kidney. This experiment shows that the rabbit liver is approximately one order of magnitude more sensitive to allyl alcohol than is rabbit kidney (Figure 4), modelling *in vitro* the same qualitative target organ susceptibility that is seen *in vivo*.

A separate experiment was conducted to determine if the tissue slice models were suited for evaluating and differentiating among tissue-specific toxicants, this time using doxorubicin as a model cardiotoxicant. This anthracycline chemotherapeutic agent has been demonstrated to cause a severe and irreversible cardiomyopathy via poorly understood mechanisms[16]. By way of contrast the toxicity of doxorubicin to kidney slices was also studied *in vitro*, since doxorubicin is not significantly nephrotoxic *in vivo* at therapeutic levels. The results of this experiment (Figure 5) indicate that the rat heart slices *in vitro* are far more sensitive to doxorubicin than are rat kidney slices, modelling *in vitro* the same qualitative target organ susceptibility that is seen *in vivo*.

FIGURE 4

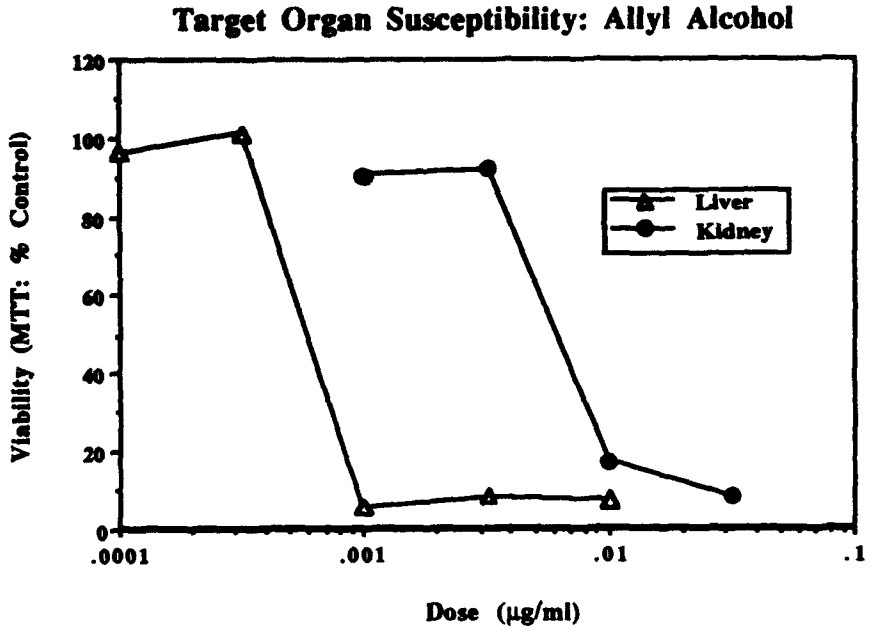
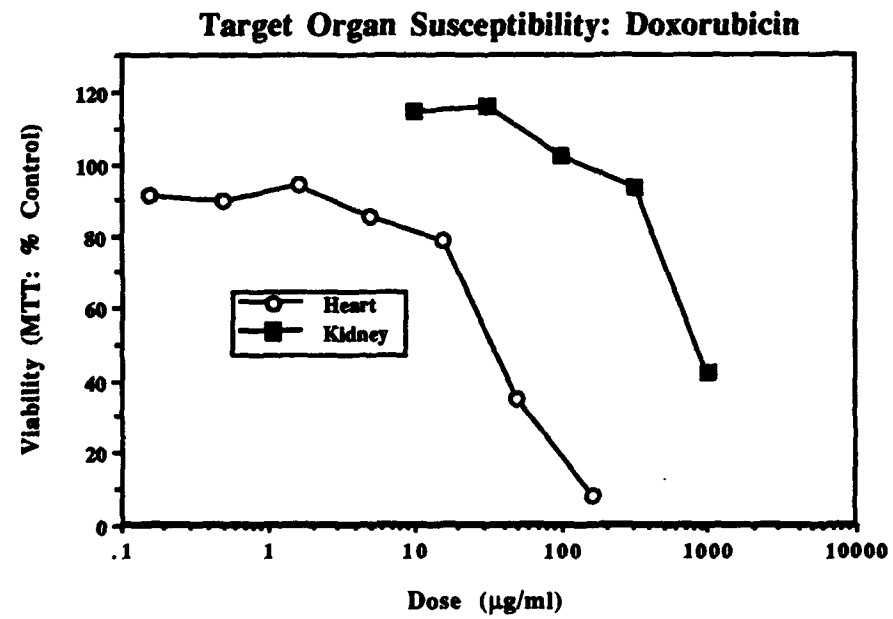


FIGURE 5



DISCUSSION

The results of these studies indicate that slices from different tissues discriminate among tissue-selective toxicants. Specifically, the cardiotoxicant doxorubicin was far more potent in heart tissues than in kidney, whereas the hepatotoxicant allyl alcohol was most toxic to liver. This model also demonstrates utility for rank-ordering the toxicity of chemically related classes of compounds, such as metals and alcohols. For example, the order of toxicity of alcohols in liver was allyl alcohol > methanol = ethanol, while in kidney slices cis-platinum was more toxic than its isomer trans-platinum, and the toxicity of metals in liver rank-order as Cd > Zn > Hg $\geq$ Cr >> Ni >> Mg. This study demonstrates that an *in vitro* tissue slice battery can provide a useful tool for rapidly identifying target organ susceptibility, chemical potency, and (through the evaluation of multiple endpoints) the mechanism of action of a wide variety of chemicals.

CONCLUSION

The utility of the precision tissue slice model is supported by the results of the studies described in this paper and by previously published studies in which this model has been used to answer related questions. Sipes, et al.[5] described the utility of this model for studying the toxicity of three different stereoisomers of dichlorobenzene, and showed that the liver slice model accurately predicted the same rank-order of toxicity for these compounds *in vitro* as is observed *in vivo*. In a recent report Wishnies et al.[17] described the utility of a liver/kidney slice co-incubation experiment to model the interactions between these two organs in producing injurious metabolites from phenacetin *in vitro*. Finally, the ability to use the slice model as a tool for evaluating cell-specific injury by combining histopathological and biochemical analyses as described by Ruegg[7] serves as yet another example of some of the unique characteristics of this *in vitro* system.

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Dr. Silber is the President of In Vitro Technologies, Inc. (IVT), located in Baltimore, MD. IVT evaluates product safety utilizing state-of-the-art *in vivo* technologies for clients in many industries including pharmaceutical, cosmetic, household product, and agrichemical companies. The company provides *in vitro* models for studying percutaneous absorption and the effect of chemicals on the eye, skin, liver, kidney, and heart. IVT is the only company nationwide that is dedicated exclusively to providing *in vitro* research and testing services.

Dr. Silber received his Public Health degree from the University of Alabama at Birmingham, after which he pursued doctoral studies in Pharmacology and Toxicology at the University of Arizona in Tucson. Dr. Silber completed his degree in the laboratories of Dr. K. Brendel utilizing *in vivo* and *in vitro* models to study pathophysiological mechanisms of renal failure with model nephrotoxicants. In 1986 Dr. Silber took a position as Senior Scientist/Toxicologist at Mary Kay Cosmetics, Inc. In this capacity he was responsible for substantiating the safety and efficacy of the company's products utilizing a combination of human, animal, and *in vitro* model systems, and establishing an *in vitro* laboratory in-house to evaluate dermal and ocular irritancy/inflammation. During this period he was elected as Vice-Chairman of the Cosmetic, Toiletry, and Fragrance Association's (CTFA) Animal Welfare Task Force, where he coordinated the CTFA Phase 1 program to evaluate *in vitro* ocular toxicity models.

In March of 1990, Dr. Silber left Mary Kay Cosmetics, Inc. to found his own *in vitro* contract research and testing laboratory, In Vitro Alternatives, Inc. (IVA). As Chairman of IVA, Dr. Silber established a variety of *in vitro* assays for predicting chemical toxicity in several model systems, including tissue slices. To be closer to his corporate clients and academic affiliates on the east coast, Dr. Silber sold IVA and established In Vitro Technologies in Baltimore, Maryland in July, 1991. IVT is located in the Technology Enterprise Center on the campus of the University of Maryland Baltimore County.

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ALTERNATIVE TESTS FOR DEVELOPMENTAL TOXICITY

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Currently, more than 25 assays have been proposed as screening tests for developmental toxicants, and these can be broken down into general categories as follows: 1) invertebrates (intact or cells in culture), 2) cell cultures (primary isolates and cell lines), 3) organ cultures, and 4) whole embryo cultures (mammalian and submammalian vertebrates). Only a few of these assays have been subjected to serious attempts at validation, either alone or as part of a test battery. Several assays are available commercially, and one has been developed into a standard assay of the American Society for Testing and Materials. Although none of these assays are sufficiently developed to serve by themselves in the risk assessment process, some may be useful for identifying mechanisms of developmental toxicity.

Alternative tests for developmental toxicity are unique because of the complexity of the whole animal systems they attempt to model. Teratogenesis involves complex interactions both within the fetal compartment and between the maternal and fetal compartments. The variety of test systems that have been proposed as alternatives for developmental toxicity testing led to the definition of alternative teratology test systems as "any system that employs test subjects other than the intact pregnant mammal"[1]. Any alternative assay for toxicity testing must have an endpoint relevant to the system being modeled. Endpoints relevant to teratogenesis include 1) differentiation of cells (e.g., biochemical or morphological differentiation, or gene activation or inactivation), 2) pattern formation (e.g., tissue or organ formation), and 3) development of complex organisms from simpler forms (e.g., invertebrates or vertebrate embryos).

Alternative teratogenesis testing systems can be broken down roughly into those that use nonmammalian species and those that use mammalian species. Nonmammalian test systems can be further divided into those that use invertebrates and those that use vertebrates. Mammalian test systems can be divided further on the basis of whether they use isolated cells, organ cultures, or whole embryo cultures. Table 1 is not comprehensive; it includes only those assays I believe are of historical importance.

TABLE 1

Alternative Teratogenesis Test Systems

I. Nonmammalian
- A. Invertebrates
  - 1) Drosophila
    - a) Embryos[2,3]
    - b) Embryonic Cells[4]
  - 2) Sea Urchins[5]
  - 3) Crickets[6]
  - 4) Planaria[7]
  - 5) Hydra[8]
- B. Vertebrates
  - 1) Fish Embryos[9]
  - 2) Amphibian Embryos[9]
    - a) Frog Embryo Tetrogensis Assay-Xenopus (FETAX)[10]
  - 3) Avian Embryos
    - a) Chick Embryo[11]
    - b) Chick Embryotoxicity Screening Test (CHEST)[12]
    - c) Chick Embryo Limb Bud Cell[13]
    - d) Chick Embryo Neural Crest Cell[14]
    - e) Chick Embryo Neural Retina Cell[15]

II. Mammalian
- A. Isolated Cells
  - 1) Established Cell Lines
    - a) Mouse Ovarian Tumor (MOT) - Lectin Binding[16]
    - b) Human Embryonal Palatal Mesenchyme (HEPM)[17]
    - c) Chinese Hamster Lung V79 - Cell-Cell Communication[18]
    - d) HEPM - Cell-Cell Communication[19]
    - e) Neuroblastoma[20]
    - f) Teratocarcinoma[21]
    - g) Monkey Kidney BSC40 - Pox Virus Morphogenesis[22]
  - 2) Primary Embryonic Cells
    - a) Rodent Limb Bud Cell Micromass[23]
    - b) Rodent Limb Bud and Midbrain Cell Micromass[24]
- B. Organ Cultures
  - 1) Rodent Tooth Bud[25]
  - 2) Rodent Palatal Shelves[26]
  - 3) Rodent Salivary Glands[27]
  - 4) Rodent Limb Buds[28]
- C. Whole Embryo Cultures
  - 1) Rodent Preimplantation Embryos[29]
  - 2) Rodent Postimplantation Embryos[30]

Nonmammalian Systems

Invertebrates

The fruit fly *Drosophila melanogaster* has been extensively characterized and is therefore a desirable model in screening tests for developmental toxicants. *Drosophila* also has extensive xenobiotic metabolizing capability. Schuler *et al.*[2] described a testing protocol and provided data that was obtained with model developmental toxicants. Female flies are mated and allowed to deposit their eggs in media containing the test compound. The flies complete metamorphosis in approximately 9-10 days. The adult flies, at least 200 per dose level, are then examined extensively for abnormalities. This assay has not been widely accepted, possibly because it requires considerable familiarity with fruit fly morphology. However, a simplified protocol was developed[3] that requires examination of only two easily recognized malformations.

Primary stem cells isolated from *Drosophila* embryos maintain the ability to differentiate into neurons and myoblasts, which can aggregate into discrete structures that resemble ganglia and myotubes. These structures are uniform in size and shape, and they can be quantitated easily with an image analyzer[4]. The basis for the *Drosophila* embryonic cell assay is then the determination of the concentration of test compound in the culture medium that inhibits formation of ganglia or myotubes by 50% relative to controls. There is good correspondence between results from this assay and from whole animal or human epidemiological studies[31]. A validation study with 100 agents found only 2 false positives (of 45 testing positive) and 4 false negatives (of 55 testing negative)[31]. One potential drawback of this assay was the expense of an image analysis system. However, image analyzers are now affordable for most laboratories. New endpoints have also been introduced, including induction of stress (or heat-shock) protein synthesis by teratogenic agents[32].

Sea urchins are a well-established model in developmental biology. Their extensive characterization and morphological simplicity make them a natural choice for screening of environmental teratogens[5]. Although the sea urchin assay was first used for identifying marine pollutants, more recent work[33] suggests attempts to use it as a screen for human developmental toxicants.

The cricket assay[6] evolved from observations of malformed wild crickets near polluted environmental sites. This assay is similar to the *Drosophila* assay, in that crickets deposit eggs in sand contaminated with the test compound. After incubation, the percent that hatch and the percent of nymphs with abnormalities are recorded. However, nothing has been published on this assay since its initial development and preliminary validation in the early 1980s. It is, presumably, no longer being pursued actively as an alternative screening test for developmental toxicants.

The planaria assay was developed on the basis of the ability of bisected flatworms to regenerate their missing portions. Abnormal morphogenetic remodeling caused by toxicant exposure is then used to assess the teratogenic potential of the toxicant. No work has been published on this assay in recent years, and it is, presumably, no longer being developed as a teratogen screening assay.

The hydra assay[8], which uses the coelenterate *Hydra attenuata*, is one of the more extensively developed alternative teratogen screening tests and is commercially available. This assay quantitates toxicity in both adult hydra and "artificial embryos" created from reaggregated cells of dissociated adults. The ratio of the concentration of test substance toxic to the adult form (A) to the concentration toxic to the developmental form (D), or A/D, then provides an assessment of teratogenic hazard. Because of their greater toxicity toward the developing organism, agents with a high A/D ratio present more of a hazard than those with a low ratio. Criticisms of the assay include whether the artificial embryo actually represents a developing form and the concept of the A/D ratio[34,35].

Vertebrates

Ecotoxicology probably provided the initial impetus for the use of fish or amphibian embryos as biomarkers for identifying environmental contaminants with the potential to cause birth defects in humans. Birge *et al.*[9] reported testing model toxicants on embryos from a variety of domestic frogs and fish. Endpoints include ability to hatch, larval mortality, and teratogenicity. One of the potential drawbacks is that fish and amphibians tend to be seasonal breeders. This problem was circumvented by using the African clawed frog *Xenopus laevis*. The frog embryo teratogenesis assay-*Xenopus* (FETAX) was developed by Dumont and co-workers[10]. The details of this assay are presented elsewhere in these proceedings (R.A. Finch *et al.*).

Avian embryos have been used longer than any other system to screen for teratogens, beginning with the pioneering work of Ridgeway and Karnofsky in the 1950s[11]. The basic procedure involves injection of test compounds into the air cell of a chick egg on gestation day 4. The embryos can then be evaluated on day 18 for malformations or incubated further to evaluate their ability to hatch. The chick embryo assay had been criticized for its extreme sensitivity (i.e., too many false positives). However, Jelinek[12] refined and standardized the assay into the chick embryotoxicity screening test (CHEST). More than 130 compounds have been evaluated with CHEST, and the assay appears to be highly predictive of mammalian teratogenicity.

Isolated chick embryo cells have also been used in proposed teratogenicity assays. Mesenchymal cells isolated from limb buds of day 4 chick embryos[13], when plated at high density in "micromass" cultures, will reaggregate and differentiate into chondrocytes. Differentiated foci can be detected and quantitated with stains, such as alcian blue, that are specific for proteoglycans. Test chemicals can be assessed for their ability to inhibit proteoglycan synthesis. Neural crest cells[14] derived from 1-day-old chick embryos will differentiate in culture into either pigment cells in the presence of fetal bovine serum or neuronal cells in the presence of horse serum. Inhibition of differentiation is assessed morphologically. Neural retina cells[15] isolated from day 6 chick embryos will reaggregate into uniformly sized spheres in suspension culture. Cultures can be assessed for cell-cell interactions by measuring reaggregate diameters. The cultures can be induced to differentiate by addition of cortisol, and one can assess differentiation and growth by assaying the neuron-specific enzyme glutamine synthetase and total protein, respectively. A preliminary assessment[15] of this assay with 22 chemicals showed a 95% concordance with in vivo mammalian teratogenicity data. This assay is currently being evaluated by an independent laboratory in a blind trial.

Mammalian Systems

Isolated Cells

Isolated mammalian cells in culture, both from established cell lines and primary isolates, are used for many developmental toxicity assays. Cell-cell interaction is an important process in developing embryos. The mouse ovarian tumor (MOT) cell assay[16] measures the effects of agents on the ability of radiolabeled MOT cells to bind to lectin-coated plastic disks. Preliminary validation studies suggest that this assay is not sensitive in identifying teratogens that act by damaging DNA, inhibiting macromolecular synthesis, or binding at specific cell-surface receptors. By contrast, the human embryonal palatal mesenchyme (HEPM) cell assay[17] measures growth inhibition and cytotoxicity in a mesenchymal cell line that originated from a day 55 human abortus. Because the MOT and HEPM cell assays are complementary, a validation study on the combined assays was conducted by two contract laboratories, using 44 coded test agents. The results[36] suggested a concordance of about 70% between the combined assay and available human

teratogenicity data. Other assays based on cell-cell interactions use gap junction communication as the endpoint. One such assay[18] quantitates the ability of 6-thioguanine (6-TG)-sensitive Chinese hamster lung V79 cells to kill co-cultured 6-TG-resistant cells by transfer of toxic metabolites via gap junctions. A similar assay with HEPM cells[19] uses autoradiography to evaluate the transfer of labeled uridine between cells via gap junctions. In both cases, teratogenicity is assessed by the ability of agents to interfere with gap junction communication. Neuroblastomas and teratocarcinomas are transformed cells that can be induced to differentiate in culture. Assays have been developed[20,21] on the basis of the ability of teratogens to inhibit this differentiation. Virus infection and replication involve a series of steps that can be likened to a developmental pathway. An assay has been developed[22] on the basis of the ability of teratogens to inhibit pox virus replication in monkey kidney BSC40 cells.

Primary limb bud mesenchymal cells isolated from day 10 mouse embryos can be cultured as high-density micromass cultures, as described above for chick embryo limb bud cells. The endpoint for the mouse limb bud cell micromass assay[23] is also the same as for the chick assay. For both assays, inhibition of chondrogenesis (measured by alcian blue staining and [^{35}S]sulfate uptake) by test agents is the basis for assessment of the agent as a developmental toxicant. Flint and Orton[24] expanded and refined the micromass assay. They isolated limb bud mesenchymal cells and midbrain neuroepithelial cells from day 12 rat embryos. Development of the limb bud cells is as above, and the midbrain cells aggregate into ganglion-like foci, which can be visualized and counted after staining with hematoxylin. Cultures of each cell type are also assessed for cytotoxicity by neutral red dye uptake. The Flint micromass assay is currently being validated in a blind, multilaboratory trial.

Organ Cultures

The suspension culture method developed by Trowell[37] allowed the culture of whole, excised embryonic organs. Cultured embryonic organs have been models in developmental biology for many years, but few organ culture systems have been evaluated as potential teratogen screens. Tooth primordia[25] can be can be excised from gestation day 14-18 mouse embryos and cultured for up to 14 days. The cultures are then fixed, sectioned, and stained. Sections are evaluated for cell death and for inhibition of growth and differentiation caused by toxicant exposure. Cultured palatal shelves[26] excised from fetal mice (day 13-14) or rats (day 15-16) undergo many of the same developmental processes as palatal shelves in vivo. Palatal shelves are also examined histologically for disruption of normal organogenesis by toxicants. Neither tooth bud nor palatal shelf culture has been developed extensively as a teratogen screening assay, but they have served as model systems for studying mechanisms of normal and abnormal organogenesis.

Fetal mouse salivary glands in culture[27] have been proposed as a screening system for teratogens. Glands excised from day 13 fetuses will undergo the normal process of lobe formation in vitro. A decrease in the number of lobes formed after 48 hours in culture is the basis for classifying agents as developmental toxicants.

Rodent limb bud culture[28] is the most extensively developed organ culture system with potential as a teratogen screen. Limb buds obtained from mouse embryos of 11-13 days in gestation will develop into recognizable paws, with most structures of the long bones and digits present. Limb buds can be cultured as suspension or submersion cultures in either serum-containing or defined media. A variety of morphological and biochemical parameters can be assayed to assess toxicity. In spite of the widespread use of the limb bud assay (over 100 publications through 1991), there is little indication that serious attempts have been made to standardize and validate limb bud culture as a teratogen screening assay. The present value of this system appears to be for studies in mechanisms of teratogenesis.

Whole Embryo Cultures

The procedures for the culture of mouse preimplantation embryos were developed in the early 1960s, and use of this system for screening for developmental toxicants was reported in the mid-1970s[29]. Mice are both time- and superovulated with hormones, and 20-40 two-cell stage embryos are collected per mouse. These embryos develop into blastocysts after 5 days in a defined medium. Blastocysts can be assessed for morphology, total cell count, and chromosomal aberrations. Further developmental potential can be assessed either by propagating blastocysts as explants that can differentiate into embryonic endoderm and ectoderm or by transferring blastocysts to receptive females where they can implant in the uterus and continue to develop. Although agents have been tested on cultured preimplantation embryos to evaluate embryotoxicity, there have been no efforts to standardize and validate this system as a teratogen screen. Because of their rapid rate of cell division and the ease with which metaphase spreads can be prepared from them, preimplantation embryos can be used for evaluating genotoxic mechanisms of embryotoxicity.

New[30] and co-workers established the contemporary method for culture of postimplantation staged rodent embryos in the 1970s. Neural-plate staged embryos from mouse (day 8) or rat (day 9) continue to develop normally for up to 48 hours when cultured in homologous serum. During this time, they will complete a significant portion of organogenesis. Effects of toxic agents can be assessed easily by gross morphology, but histological and biochemical parameters can be evaluated as well. Several groups (e.g., Govers *et al.*[39]) are attempting to standardize and validate postimplantation rodent embryo culture as a teratogen screening assay. Because human serum supports growth and development of cultured embryos, Chatot *et al.*[39] have suggested that cultured embryos may serve as biomarkers for screening the serum of women for factors that may result in adverse reproductive outcomes. Finally, postimplantation embryo culture is an exquisite system for studying mechanisms such as the role of adrenergic agents in situs inversus[40].

Validation of Alternative Tests for Developmental Toxicity

Validations are based on comparisons of assay performance against a standard list of teratogens and nonteratogens[41]. Because these teratogenicity data are drawn from multiple species, protocols, and endpoints, it is difficult to interpret what "validation" against such an assortment means. The standard criteria required for an alternative developmental toxicity assay for assessing human reproductive hazards must be clearly defined.

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THOMAS J. FLYNN, Ph.D.

Dr. Flynn received his Ph.D. in biochemistry from Temple University Medical School under Dr. Ronald Pieringer. His thesis research focused on the effects of thyroid hormones on glycolipid metabolism in developing rat brain. He also isolated and identified a novel sulfated glycero-glycolipid in rat brain. Dr. Flynn moved to the Biology Department at Temple University where he did his post-doctoral research under Dr. Nina Hillman. There he conducted studies on lipid metabolism in cultured pre-implantation mouse embryos and showed that they accumulate triglycerides prior to hatching of the blastocyst. Dr. Flynn joined the FDA in 1980 as a Staff Fellow and presently works there as a research scientist in the Division of Toxicological Studies in the Center for Food Safety and Applied Nutrition. Dr. Flynn's work with the FDA has focused on the identification and evaluation of *in vitro* alternative tests for developmental toxicity. He has used cultured post-implantation rat embryos to study mechanisms of teratogenesis of well known teratogens such as retinoic acid and thalidomide. He is presently evaluating embryos cultured in human serum specimens as biomarkers for identifying human women with nutritional deficiencies which may result in reproductive failure. Dr. Flynn is a member of the American Chemical Society, the American Association for the Advancement of Science, the Society of Toxicology, the Teratology Society and the Tissue Culture Association. In 1990, he received the FDA's Commendable Service Award for sustained superior performance in studies to develop *in vitro* alternatives in teratology and developmental biology.

IN VITRO ASSAYS FOR MUSCLE IRRITATION

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Abstract. In vitro target cell assays have been used either as predictors or substitutes for muscle irritation assays of medical device materials. In vitro systems model the bioavailability of chemical substances in vivo in terms of chemical migration, solute partitioning, solute solubility, protein binding and other factors affecting target cell dose. In vitro assays separate toxicological and pharmacological aspects of the biological response. This has contributed to the international acceptance and standardization of these models in the medical device industry.

INTRODUCTION

Medical devices have been tested for biological reactivity for more than 30 years. The initial concern was whether the new plastic materials being used for disposable syringes and catheters would adversely effect the solutions, drugs and tissues in the practice of medical care. Designing appropriate biological assays focused on extracting the plastics with various nontoxic solvents (saline, saline with 5% ethanol, polyethylene glycol 400 and vegetable oil) and testing the extract for biological safety or testing the solid material directly in an animal model. The safety tests were generally adaptations of those tests commonly used for pure chemicals, namely acute systemic toxicity and irritation potential by cutaneous, intradermal and intramuscular routes. In the ensuing years, biological reactivity testing changed from an empirical view to a mechanistic based approach using advances in biology, chemistry and polymer science and technology. This article reviews the science of that transition and the rationale for the adaptation of cytotoxicity assays as an alternative to in vivo biological reactivity tests.

MUSCLE IRRITATION ASSAY

The in vivo biological reactivity tests, namely the systemic injection, intracutaneous irritation and intramuscular implant assays, are described in the US Pharmacopeia.[1] Experience has shown that the intramuscular irritation test was the most sensitive of the three assays. This assay involves the insertion of a small sample of solid material in the paravertebral muscles using a trocar and needle (Fig. 1). Insertion of the needle into tissue initiates a wound healing reaction characterized by an influx of neutrophils and macrophages, which cleanup the microorganisms, dead cells and other debris (Fig. 2). This is followed by an influx of fibroblasts that repair the damage with a mortar of collagen. The tissue reactivity (Fig. 3) is evaluated by the amount of encapsulation of the test article. The size of the wound and amount of scar tissue is dependent on the amount of tissue damage. That is, any chemicals extracted by the tissue from the test sample can potentially cause kill cells if the cellular concentration attains a toxic dose.

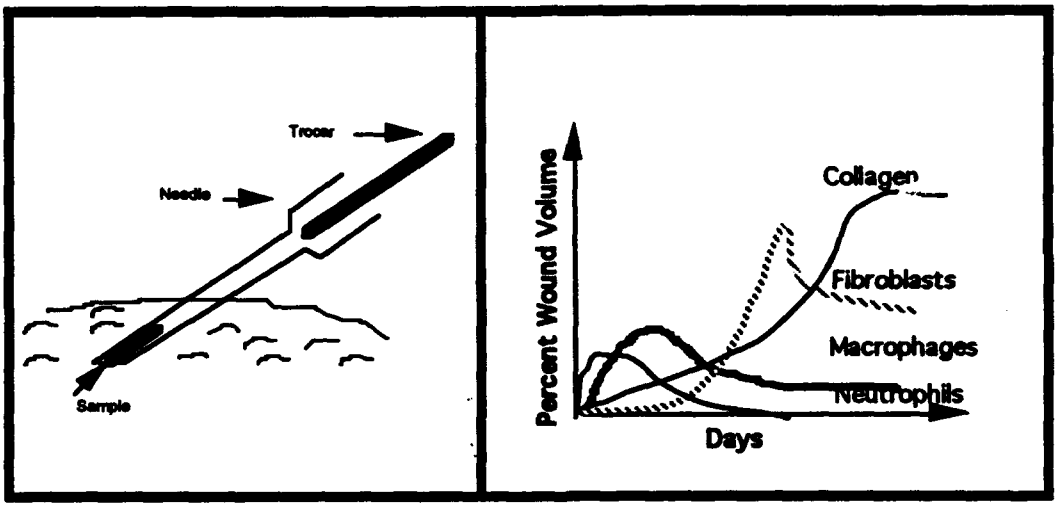

Fig. I. Trocar method of soft tissue implantation.

Fig. 2. Normal wound healing.

Fig. 3. Muscular irritation assay of a reactive plastic (upper right) illustrating fibrotic wound healing.

The physicochemical mechanisms effecting intramuscular irritation are listed in Table 1. The biological reactivity between the implanted test sample and adjacent tissue is based on the target cell dose of the leachable chemicals from the implant. The quantity of chemical(s) is affected by a partitioning of the chemical in the aqueous and lipoidal solvents that constitute the tissue. Binding of lipid-soluble chemicals by the hydrophobic region of proteins enhance solubility. There is a slow, continuous dissociation of the agent into the aqueous phase as determined by its solubility and dissociation rate from the proteins. The limited metabolic activity and lack of dilution by the vascular system and tissues also contribute to increase the target cell dose by affecting detoxification and elimination rates.

Table 1. Intramuscular Irritation Assays

Target Cell Dose
Chemical partitioning affected by water and fat content of tissue
Limited metabolic activity
Limited dilution

BIOAVAILABILITY

The physicochemical mechanisms effecting the bioavailability[2,3] or release of the chemical(s) from the implant are listed in Table 2. The total available pool is the amount of chemical(s) initially present in the test article. If the chemical is chemically bound to the polymer, it would not be free to migrate out of the material. Any unbound chemical(s) could migrate at a rate dependent upon size, configuration, electronic charge, etc. The migration rate is closely linked with partitioning of the chemical(s) within the bulk phase of the material, to the surface and into the solvent phase of the tissue. The amount of chemical(s) extracting into the tissue varies with its solubility in the tissue.

Table 2. Mechanisms Limiting the Bioavailability of Leachable Chemicals

Total available pool
Migration rate
Partitioning
Solubility

The dynamics effecting bioavailability are further illustrated in Fig. 4. Any unbound chemical(s) in the bulk phase of the material can migrate within the bulk phase, to the surface and then out into the tissue. The rate and amount over time are effected by the partitioning among the three phases--bulk, surface or tissue--and the solubility limit in each phase. Figuratively, one can describe these dynamics as a student moving from home (home = bulk phase) to college (college campus = surface). If the student is not happy at college, he/she moves back home (a case of no migration). Alternatively, the student graduates from college (partitioning at the surface) and gets a job in a city (city = tissue). Any additives to a plastic are theoretically expected to remain in the plastic because the plastic itself is a water insoluble (i.e., lipophilic), solid substance. Thus, the total available pool is often much greater than the amount of chemical that is

extractable in a non-lipophilic media. Additionally, the hydrophilic nature of tissue itself would limit the solubility of any lipophilic chemicals that could potentially migrate.

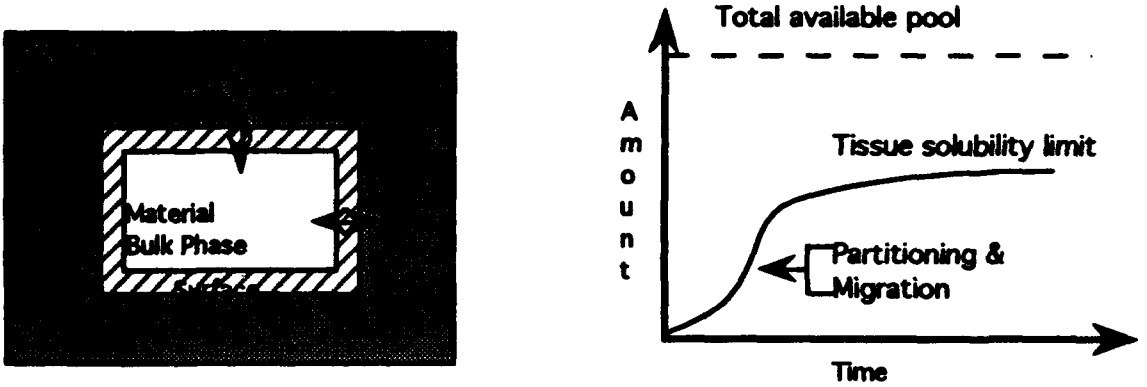

Fig. 4. Dynamics of the bioavailability of extractive chemical(s) from plastic materials implanted in tissue.

The migration of ethylene oxide, a sterilizing gas, from a medical device illustrates the bioavailability of extractive chemical(s) to tissue cells. The amount of residual ethylene oxide in the material after sterilization varies with the thickness of the material and elapsed time since sterilization among other factors. Thicker material will absorb a greater amount of the sterilant during the sterilization process. If the material is then placed in an open environment or tissue, the free chemical (i.e., ethylene oxide) will migrate out of the bulk phase of the material into the surrounding environment. In the case of cells or tissue, the concentration of free chemical in each cell will determine the biological effect of the extractable ethylene oxide. The target cell dose effect of residual ethylene oxide in polystyrene, for example, resulted in substantial variance background mutagenicity rates.[4]

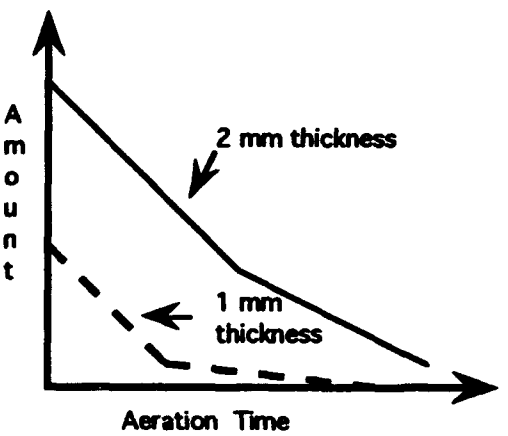

Fig. 5. Residual ethylene oxide variance with material thickness

IN VITRO ASSAYS

The challenge of developing an in vitro alternative to the intramuscular irritation assay initially involved selecting the appropriate parameter

to model. Fig. 6 outlines the pharmacological effects of a toxin or immune stimulus. The primary event is cellular injury resulting in a change in the permeability of the cell membrane or the lysis of the cell. Either event is followed by a release of pharmacological agents, hemodynamic effects, etc. Thus, as a first approximation, an in vitro cell injury model gives the potential for the secondary pharmacodynamic effects. Sources of variance as dilution and metabolism are eliminated in an in vitro model by a defined volume of cell media, choice of cell line and number of cells. This results in a controlled dose of test substance per individual cell in vitro as compared to the unknown target cell dose of in vivo assays. A fibroblast cell line was selected for the in vitro model to evaluate would healing (Fig. 2), an indirect measure of the amount of cell damage. These considerations led to an in vitro model using a fibroblast cell line in a near confluent culture and measurement of the cytotoxic effect of leachables from the polymeric materials.

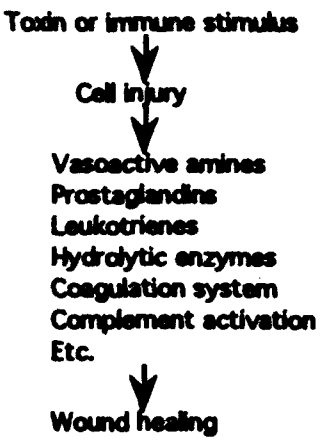

Fig. 6. Biological effects of a toxic or immune stimulus.

Three different in vitro cytotoxicity models have been widely adapted by the medical device industry.[5,6] The models differ in the preparation of the test sample and the mode of exposure. In the direct contact model, the test sample is placed on top of a monolayer of fibroblasts (Fig. 7). Chemicals are extracted from

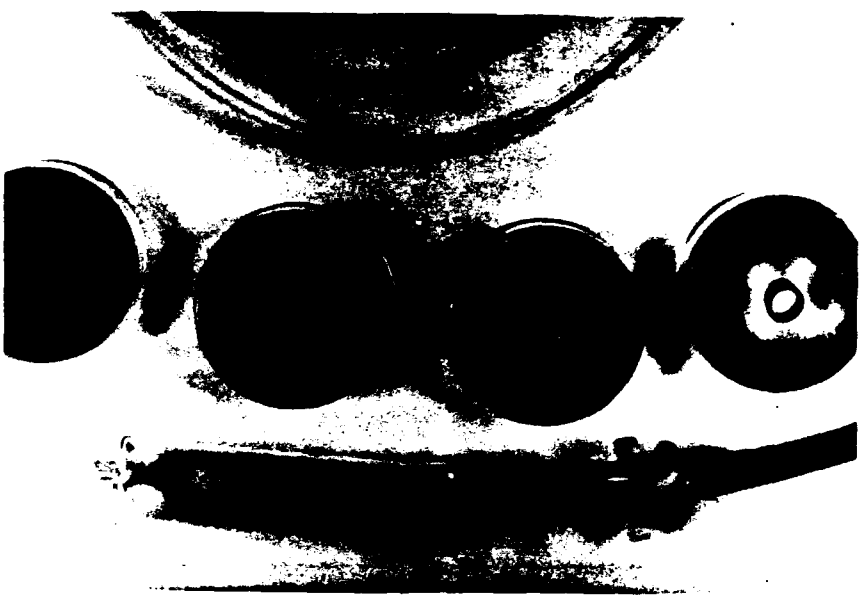

Fig. 7. In vitro direct contact assay.

the sample by the solvent effect of the culture medium and added serum. At the end of a 24 or 48 hour exposure period, the culture medium is removed and the remaining viable cells are treated with a histological stain. The culture dish on the right side of Fig. 7 shows cytotoxicity to the cross section of tubing as evident by the lack of stained cells surrounding the sample. Cytotoxicity was caused by extraction of a relatively low molecular weight organotin compound that was used as a stabilizer in the plastic formulation. In the agar diffusion assay (Fig. 8), a thin layer of agar or agarose mixed in culture media separates the test

Fig. 8. In vitro agar diffusion assay.

sample from the cellular monolayer. The agar functions to protect the cells from physical damage by movements of the test sample and creates a somewhat concentrated diffusion zone around the sample. Cytotoxicity is evaluated by the lack of staining of dead cells with a vital stain. In the elution test, the test sample is extracted in a nontoxic vehicle and then exposed to the cells. This method offers flexibility in the choice of extractant, and time, temperature and duration of extraction. Cytotoxicity is evaluated by the density of cells remaining after a 24 or 48 hour exposure of the cells to the extract. The detection limit of these assays can be lowered (i.e., made more sensitive) by reducing the cell number, using cells with a larger cell volume, using cells in the log growth phase rather than at near confluency and extending the exposure period. These variables improve sensitivity by altering the dose per cell or increasing the environmental stress on the cell. The target cell dose in the three assays is dependent on the total available pool of cytotoxin in the test sample, the rate of migration from the bulk and surface of the material, partitioning from the sample surface into the culture medium and solubility in the culture medium and cells. These are the same factors effecting bioavailability in vivo following implantation.

CONCLUSION

Two decades of success in the application of in vitro models for testing medical devices can be attributed to matching the in vivo target cell type and approximating the bioavailability of the cytotoxin. The in vitro models described

herein accurately predict the acute in vivo biological reactivity of a wide variety of synthetic polymers and plastics. These models focus on reversible toxicological events free of pharmacological mediators. Adapting a mechanistic approach on the types of cytotoxins and their bioavailability during clinical use of the medical devices has ensured the validity, reproducibility, accuracy and predictability of the in vitro assays.

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APPLICATIONS OF LIVER AND KIDNEY CELL SYSTEMS THAT CAN REDUCE ANIMAL USAGE

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ABSTRACT

Primary cell and tissue culture systems from animal and human organs have practical value for a variety of toxicological and pharmacological applications. Examples of the validation and use of hepatocyte and renal proximal tubule systems as screens are cited in this presentation. Significant reductions in animal use and associated testing costs are possible, as long as the data provided are mechanistically based and relevant to *in vivo* response.

INTRODUCTION

Interest in the development and use of primary cell systems for toxicological and pharmacological applications has increased dramatically over the last 15 years.[1,2] We surveyed abstracts from past Society of Toxicology annual meetings for reports on purely *in vitro* cell or tissue culture studies and found an increase from 11% of the total presentations in 1980 to 24% in 1990; other studies with subcellular preparations or cells from animals treated first with the toxicant added a few more percentage points. Many of these studies were developmental or involved basic research on toxic mechanisms, but technology has progressed to the point where some of these systems are being applied to a variety of toxicological and pharmacological problems.

The purpose of this presentation is to provide examples of the kinds of information obtainable with primary cell and tissue systems and their potential applications. Most of our work has been done with isolated hepatocytes (HEP) and renal proximal tubule suspensions (RPT) because they are common targets of drug- and chemical-related toxicity and because they play central roles in the disposition and excretion of foreign compounds after absorption.

METHODS AND MATERIALS

ISOLATION PROCEDURES

Detailed methods of isolating HEP from either whole liver or biopsy specimens by collagenase perfusion are described elsewhere.[3-5] The viability of the freshly isolated cells was assessed by trypan blue exclusion; only preparations with >80% viable cells were used. Erythrocytes were harvested from heparinized or EDTA-treated whole blood by centrifugation and stored in culture medium at 4°C overnight; they were recentrifuged and resuspended in culture medium at ~1.6 hemoglobin/dl before use. RPT were isolated *in situ* by a modified collagenase perfusion method in which deferoxamine was added to the perfusate to reduce oxidative damage to cells during isolation.[6] When a section of a baboon kidney was received at the laboratory for use, the perfusion was conducted through a cannula inserted into a vessel on the outer surface and the procedures thereafter were the same.[7] RPT viability was determined primarily by measuring nystatin-stimulated oxygen consumption.[6,7] Only preparations with >35% stimulation retained enough viability throughout the experiment (4 hr or longer) to be used.

CULTURE CONDITIONS

Both HEP suspension (1×10^6 viable cells/ml of culture medium) and monolayer ($\sim 0.25 \times 10^6$ viable cells/ml) cultures were used, depending on the experimental objectives. A coculture system—HEP attached to culture dishes with erythrocytes suspended in the medium—was also used.[8] The culture medium was hormone-supplemented Waymouth's 752/1 containing either 0.2 or 2.0% bovine serum albumin (BSA).[5,7-9] RPT were incubated in suspension in the same medium containing 2% BSA at a protein level of 0.5 mg/ml with shaking under essentially the same experimental conditions.[7]

All incubations were conducted at 37°C under an atmosphere containing 20% or higher O_2, 5% CO_2, and the balance N_2. HEP and RPT suspensions were shaken at 70 osc/min in airtight Erlenmeyer flasks with sidearms for serial sampling.

ASSAYS

Amphetamine (AMP) metabolism was monitored by using D-[7-^{14}C]AMP sulfate (Research Products Intl., Mt. Prospect, IL), and high-pressure liquid chromatography was used for metabolite separation.[5] Acetaminophen (APAP)-protein adducts were determined immunochemically after electrophoretic separation of the proteins by using an affinity-purified anti-APAP antibody developed in Dr. E. A. Khairallah's laboratory at the University of Connecticut (Storrs).[10,11] Trichloroethylene (TCY; [U-^{14}C] radiolabel from Amersham, Arlington Heights, IL) and its metabolites were assayed by gas chromatography.[9] The MTT assay was conducted as described spectrophotometrically using a Dynatech microplate reader.[12] In cyanide (CN) antidote experiments,

cytotoxicity was monitored by determining cell adenosine triphosphate (ATP) levels using the lucerifin-luceriferase assay after aspiration of the erythrocyte-containing culture medium and solubilization of HEP.[8] All chemicals and other reagents were purchased from established commercial suppliers and used without further purification.

RESULTS AND DISCUSSION

USE FOR COMPARATIVE METABOLISM

Early work in this laboratory focused on the value of HEP systems as *in vitro* models for interspecies comparisons of drug and chemical metabolism. Studies on AMP and TCY metabolism provide clear examples of the ways *in vitro* data can be used both prospectively to select the most representative animal models for absorption/distribution/metabolism/excretion (ADME), pharmacokinetic, and toxicity studies and retrospectively to help clarify the significance for humans of findings in laboratory animals.[5,9]

AMP metabolism occurs in the liver by two principal pathways: (1) ring hydroxylation, which forms p-hydroxy-AMP; and (2) oxidative deamination, which forms phenylacetone, benzoic acid, and hippuric acid as principal metabolites. *In vivo*, the rat had been shown to metabolize the drug primarily via ring hydroxylation, whereas in rabbits, dogs, squirrel monkeys, and humans, oxidative deamination is the principal pathway (Figure 1a). These findings were reproduced *in vitro* (Figure 1b). Also, we found a good inverse rank correlation between the rates of hepatic AMP metabolism and the amount of AMP excreted unchanged in animal and clinical studies. Each species was unique in the way it handled AMP (or, for that matter, all other drugs and chemicals we have examined), but the rat, the most commonly used species in toxicity and metabolic disposition studies, was clearly not representative of human exposure in this case.

These results are instructive from another standpoint. Imagine that you are designing a test strategy to support an Investigational New Drug Application for amphetamine without the benefit of *in vivo* data. If you conduct ADME and pharmacokinetic studies in rats, you have chosen the wrong species as a model for the way humans handle this drug. If you conduct the same experiments with dogs, you will get entirely different results. Without human studies, you would not know which species was more representative. Preliminary testing in hepatocytes from both species and comparison with human *in vitro* data allow us to determine the more appropriate species for pharmacokinetic/ADME studies and to weight our toxicological data accordingly before human exposure.

In vivo studies in several laboratories have demonstrated that TCY is hepatocarcinogenic in the mouse but not in the rat. Because this compound is a proven hepatocarcinogen in an experimental model, regulatory agencies are highly concerned

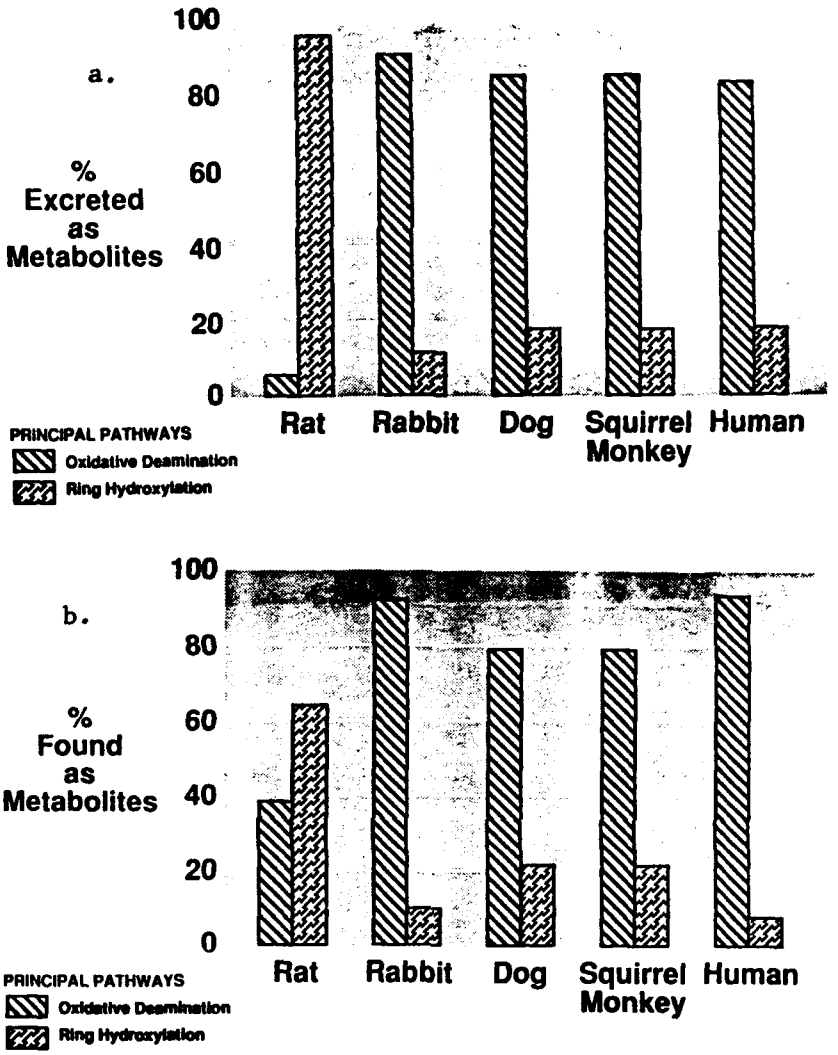

Figure 1. AMP metabolism. a. *In vivo* 24- or 48-hour urine collection. b. *In vitro* 4-hour incubation with HEP.

about the consequences of human exposure in the workplace and the environment. A metabolite, trichloroacetic acid (TCA), appears to be primarily responsible. The mouse metabolizes TCY four times faster than the rat at comparable doses and produces correspondingly larger amounts of TCA.

Comparative metabolism studies in rat and human hepatocytes under identical experimental conditions were proposed as a way of determining whether humans are more or less at risk from internally formed TCA. Incubation of TCY with these cells produced trichloroethanol, its glucuronide conjugate, and TCA as the principal metabolites in roughly the same proportions as found *in vivo* in these species. Much lower levels of TCA were present in the human cell incubations compared with those in

rat HEP, both in total amount and relative to trichloroethanol plus conjugate (Figure 2). Because the same principal metabolites were formed as *in vivo*, we may conclude that, all other factors being equal, humans are less prone than rats, a known resistant species, to develop hepatocarcinogenicity from TCY exposures.

USE FOR CYTOTOXICITY SCREENING

Several laboratories are exploring the potential value of primary cell cultures as cytotoxicity screens to predict the general or organ-specific toxicity of previously untested compounds. We have conducted a series of ongoing studies aimed at defining the context within which these screens can be used (i.e., what useful information they can provide). In early work we showed that the cytotoxic potentials of chlorinated aliphatics in HEP suspensions were closely correlated with *in vivo* toxicity for these chemicals when corrections were made for differences in expiration and volatility in the *in vitro* and *in vivo* systems.[13] The cytotoxicity of dinitrotoluenes in HEP suspensions also correlated well with their hepatotoxic potential in rats, which toxicity based on molecular orbital calculations is related to the electronic rather than the physicochemical properties of the compounds.[14] Cytotoxic potentials determined for cephalosporins and aminoglycosides in RPT systems show very good rank correspondence to the nephrotoxic potentials of these classes of antibiotics.[15,16] These results and those from other laboratories suggest that HEP and RPT are practical models for estimating the toxic potencies of structurally related compounds to target cells for those chemical classes. Occasional exceptions and anomalies to this "rule" that have appeared in the literature need to be explained, and the investigator must often balance the imprecision of the inverse ranking orders of some individual compounds in the series against the overall correlative value (for which $r^2 > 0.80$ in the cases cited).

If these systems are useful for ranking the cytotoxic potency of untested compounds relative to some reference compounds within the series, they must also have value for detecting interspecies differences in response. We obtained insight on this point in two separate studies. In one, we determined the cytotoxic potentials of 9 compounds from the Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC) list in a rat HEP assay using MTT-tetrazolium dye reduction as the indicator and compared the results with available *in vivo* data.[12] The cytotoxic potentials correlated better with rat oral LD_{50} values than did circulating concentrations of the compounds known to cause toxicity in humans (Figure 3). Although the comparison cannot be made more rigorous in the absence of rat blood or tissue concentrations at lethal doses, a reasonable expectation is that rat HEP should predict rat toxicity more accurately than human toxicity, and in this sense the results support that expectation. Others have tested 10 compounds from the same list *in vitro* and demonstrated that human hepatocytes predicted acute toxicity in humans more accurately than either rat hepatocytes or mouse 3T3 cells.[17]

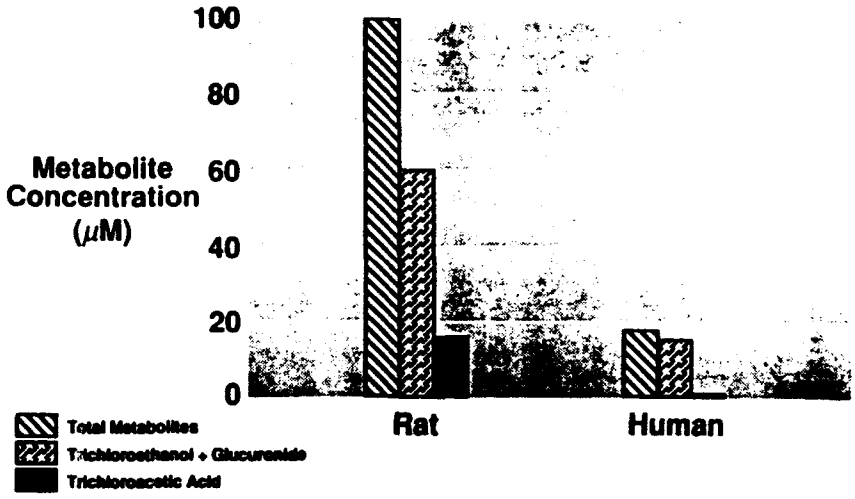

Figure 2. TCY metabolism. *In vitro* 4-hour incubation with HEP.

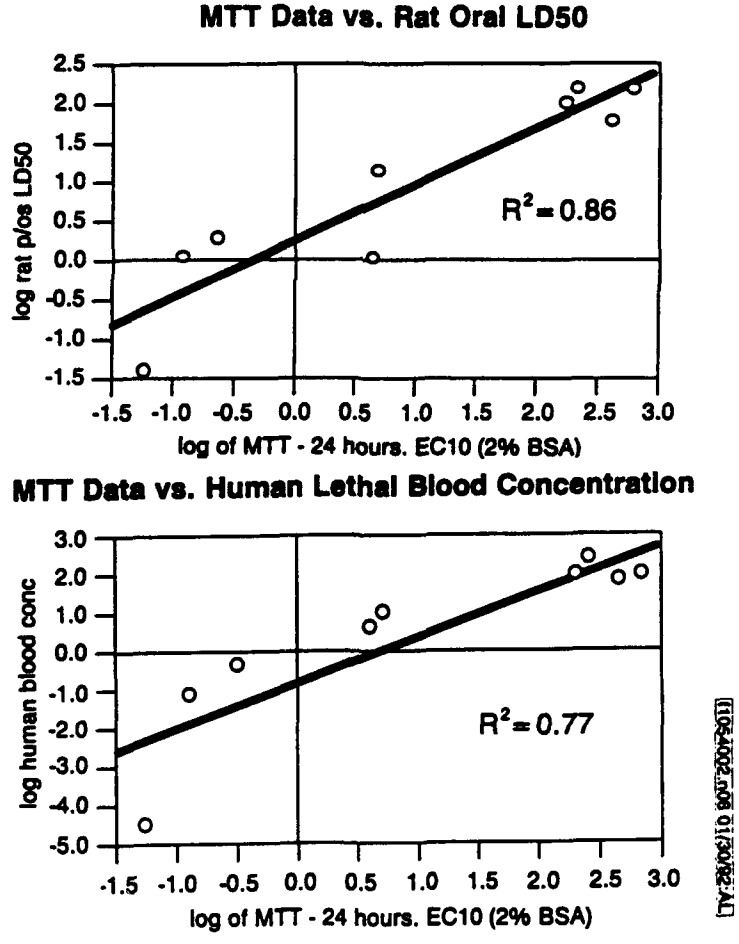

Figure 3. Effective concentration for 10% change in MTT response for MEIC compounds in 24-hour HEP cultures compared with *in vivo* response.

Using acetaminophen (APAP) as a test compound, we and others had reported earlier that mouse, hamster, rat, rabbit, and dog HEP show the same relative susceptibility to the drug as is found *in vivo*.[18,19] These *in vitro* data also suggest that humans are relatively resistant compared with other species (Figure 4). APAP binds to cell proteins at toxic levels, and the same protein-bound adducts were found immunochemically in mouse and human HEP as *in vivo*.[7] (The latter result was obtained with a liver from an accidental fatality after APAP overdose.) Whereas RPT from CD-1 mice showed a binding pattern similar to that in liver and kidney, no protein adducts were found in incubations with rat tubules (as with intact kidney in *in vivo* experiments) or with those from a baboon (human kidney being unavailable at the time).[7] While the differences in response remain to be clarified, they were profound among these species. Kidney failure from APAP overdose is of more serious concern to some clinicians than liver injury, and the relevance of the mouse, which some investigators use as a model, for evaluating various treatments to reverse APAP toxicity in the human is questionable at this point.

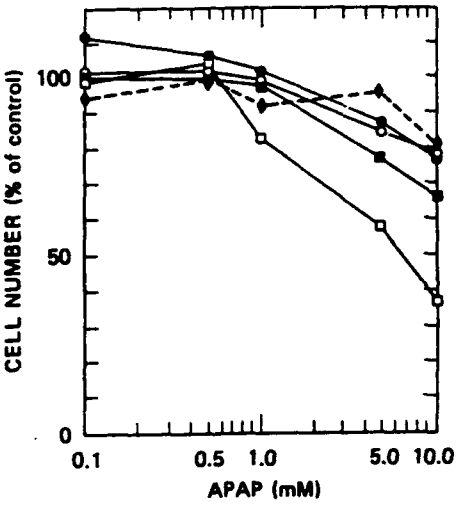

Figure 4. APAP-induced cytotoxicity in 24-hour HEP cultures. -□-□- hamster; -■-■- dog; -O-O- rat; -●-●- rabbit; -♦-♦- human.

USE AS SCREENS FOR PHARMACOLOGICAL ACTIVITY

The utility of primary cell systems for screening for pharmacological activity *in vitro* is also becoming increasingly evident. For example, the U. S. Army Medical Research and Development Command was interested in a screening system that would allow selection of the best prospective anticyanide agents for further evaluation from among a large number of candidate compounds. The currently recommended treatment for cyanide (CN) poisoning in this country is a combination of sodium nitrite ($NaNO_2$) and sodium thiosulfate ($Na_2S_2O_3$); cobalt compounds are used in Europe. To provide optimal versatility in the screen, we developed a coincubation system comprised of HEP monolayer cultures (as both target cells for CN action and a source of the enzyme rhodanese, which uses $Na_2S_2O_3$ as a substrate for CN detoxification) and

erythrocyte suspensions (which provide hemoglobin for nitrite-induced cyanmethemoglobin formation). Cobalt complexes CN directly and nonenzymatically.

In the coincubation system and under the experimental conditions used, CN-induced ATP depression occurs only in the HEP and is a convenient indicator of cytotoxicity. $NaNO_2$ and $Na_2S_2O_3$ were each partially effective and their combination was most effective in reversing this effect in rat cell cultures (Figure 5). This result is known to occur in CN-treated animals *in vivo*.

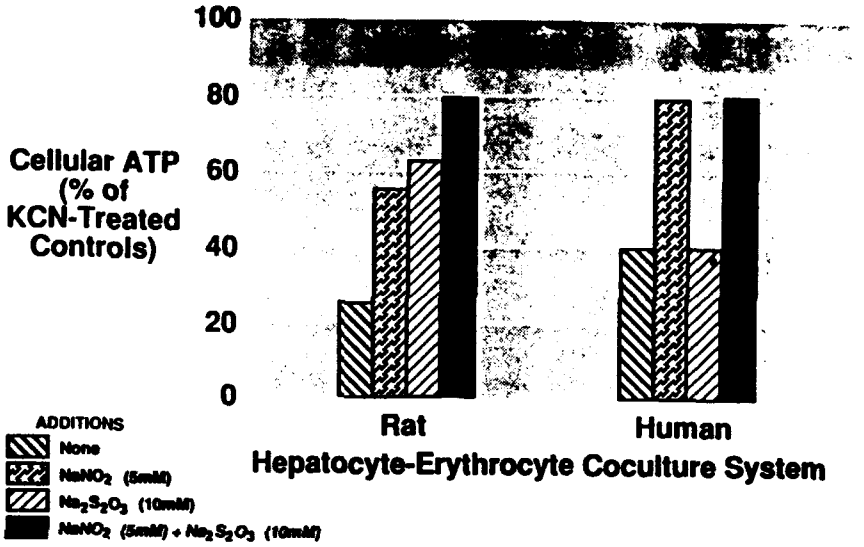

Figure 5. Antidote reversal of CN-induced cytotoxicity.

In contrast, $NaNO_2$ but not $Na_2S_2O_3$ reversed ATP depression in the human cell culture and the effectiveness of the combination was totally dependent on the $NaNO_2$ component (Figure 5).[20] The apparent reduced effectiveness of $Na_2S_2O_3$ in the human cell system corresponds to the known lower rhodanese activity in human compared with rat liver. These results both verify the value of this approach to screening CN antidotes and encourage a closer examination of the merits of $Na_2S_2O_3$ for human therapy.

Another pharmacological application for HEP cultures has been in screening drugs and drug-ionophore combinations for treating Fe overload in blood-transfused β-thalassemia patients.[21] Our laboratory has also developed a promising lipocyte assay for screening drug candidates with potential effectiveness in treating hepatic fibrosis. Reaggregate brain cell and heart cell cultures from both animal and human tissue are being evaluated for both pharmacological and toxicological applications.

CONCLUSIONS

These examples demonstrate the benefits of using primary cell screens to reduce animal use and to provide data of toxicological and pharmacological significance. One rat liver or a pair of kidneys provides enough cells or tissue for 30 to 40 separate experimental flasks for screening or mechanistic studies, with a corresponding reduction in the number of animals and test costs required to obtain the necessary information. In addition, a strong mechanistic basis for using these systems as screens for acute toxicity and metabolism has been developed in many laboratories. Toxicologists are beginning to realize that information from these screens is as important as that obtained from mutagenicity and carcinogenicity screens because primary cell screens provide key data for making decisions at critical junctures in the developmental process, avoiding the wasteful use of animals for testing less than optimal compounds. The use of such data for extrapolations to safe dose or exposure levels for humans is an ultimate, additional goal that will be achieved when *in vitro* data are routinely incorporated into physiologically based pharmacokinetic models for risk assessments.

Proper interpretation of *in vitro* results, however, is not always as straightforward as in the examples cited. For instance, renal tubule preparations may be acceptable screens for assessing metal- and antibiotic-induced nephrotoxic potential, but several compounds are known to cause kidney toxicity via metabolites formed by the liver. The ability to estimate the nephrotoxic potentials of such compounds from *in vitro* data requires the use of coincubation systems and conditions that accurately model *in vivo* organ specificity and potency. Progress in this direction is occurring in a few laboratories. The hepatocyte-erythrocyte system was the first to show that primary cell coincubation systems could mimic *in vivo* response and potency with pharmacologically active drugs. Another classic example is the Ames test, in which liver S-9 postmitochondrial fraction is added to *Salmonella* bacteria to assess whether metabolic activation of the test compound is required for mutagenesis. We can envision similar strategies with kidney RPT and cells from other potential target organs supplemented with S-9 or intact liver cells.

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Session VII: Poster Presentations

Co-Chairs: MAJ Billy Howard and Dr. Brennie Hackley

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Dr. Howard lists numerous presentations to his credit and a recent publication (Nov-Dec 1990) is "The Cardiovascular Response of Sheep to Tiletamine-Zolazepam and Butorphanol Tartrate Anesthesia," Veterinary Surgery 19(6):461-167.

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STRUCTURE-ACTIVITY PREDICTIONS AS ALTERNATIVES TO ANIMAL TESTS

Kurt Enslein, HDi, Rochester NY

Animal tests that have been performed in the past can form the basis for equations modeling specific toxicity endpoints. These equations can then be used to predict the same endpoints for chemicals for which the bioassays have not been performed. These principles of structure-activity relationships (SAR)[1,2] have been implemented in the TOPKAT program. SAR models exist for the following mammalian toxicity endpoints:

Rat oral LD_{50}[3]
Rat oral maximum tolerated dose[4]
Rabbit skin irritation (Draize)[5]
Rabbit eye irritation (Draize)[6]
Carcinogenicity (2-species, 2-sex)[7]
Teratogenicity (frank malformations)[8]
Rat oral chronic LOAEL[9]
Mouse inhalation LC_{50}[10]

Data used for the development of these models are carefully screened and, often, "harmonized" to make them suitable for the stringent requirements of predictive equations.

Estimates produced by the TOPKAT program can be internally validated through technology provided within it. This is a crucial requirement, and differentiates the TOPKAT program from other predictive systems.

The TOPKAT SAR models have found use in both regulatory as well as industrial applications. Regulatory applications have included submissions as part of PreManufacturing Notifications (PMN) to the US EPA, the examination of impurities in marketed drugs, the complementing of Material Safety Data Sheets (MSDS) with estimated values in place of actual assay data, the evaluation of intermediates of chemical manufacture, the estimation of rat oral LD_{50} values for experimental chemicals, and the partial fulfillment of the FDA Environmental Assessment requirements for Phase I Investigational New Drug (IND) studies.

In addition to several of the above examples which also have found application in industry, TOPKAT has been used for the prioritization of discovery candidates, the reduction of toxic effects for developmental compounds, the cross-validation of bioassays, dose ranging for bioassays, and performing toxicity experiments in calculo.

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Using Theoretical Descriptors in Quantitative Structure Activity Relationships

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ABSTRACT

Quantitative Structure Activity Relationships (QSAR) have been used successfully in the past to develop predictive equations for numerous biological and physicochemical properties. Linear Solvation Energy Relationships (LSER), a subset of QSAR, have been used by Kamlet and Taft to correlate over 100 solute/solvent dependent interactions with a set of empirically derived descriptors. A major difficulty with this approach has been the use of these empirically determined parameters. A new approach based upon the generalized LSER approach has been developed. This technique, called Theoretical LSER (TLSER), uses only structural and quantum chemically derived descriptors. The TLSER has been shown to correlate very highly with over twenty physical and toxicological activities. The utility of this method to the a priori prediction of chemical and biological properties will be discussed.

1. Introduction

Quantitative Structure Activity Relationships (QSAR) have been used extensively in correlating structural features to physical, biological and toxicological properties. The basic tenet of QSAR is that there is a connection between the microscopic (molecular structure) and the macroscopic (empirical) properties. Further, this connection can be used to predict empirical properties directly from the molecular structure. This relationship was first quantized by Hammett, who developed the *Linear Free Energy Relationship* (LFER).

1.1 Linear Solvation Energy Relationships

Based on the concept of Hammett, Kamlet and Taft developed a methodology for developing Free Energy Relationships based on solute/solvent interactions.[1-4] This relationship, defined as the *General Linear Solvation Energy Relationship*, is shown in equation 1.

$$LOG\ Property = Steric + Polarizability + Hydrogen\ Bonding \quad (1)$$

In this way, a given property can be described as a linear expression consisting of contributions due to steric factors, polarization and polarizability factors, and hydrogen bonding factors. In the multiple solute - single solvent system, the specific terms are: Steric- Molar Volume (V_m), Polarizability- spectroscopically determined polarizability (π^*), Hydrogen Bonding- spectroscopically determined acidity and basicity terms (α and β). In practice, not all four terms are required for every relationship. The coefficients and associated statistical t-scores can be used to gauge the importance of each descriptor for every property correlation. In this way the LSER can be used to infer insight to solute/solvent interactions.

One major difficulty of this approach has been the nature of the descriptors. All are empirically determined, therefore reducing the usefulness of this approach for *a priori* predictions. Some attempts have been made correlating more fundamental structural and electronic descriptors with the Kamlet-Taft solvatochromic parameters with moderate degrees of success.[5]

1.2 Applications of Theoretical Chemistry

Theoretical chemistry has been used in the past to supply structural and electronic descriptors for QSAR and QSAR-like equations. In this way, empirically derived descriptors can be replaced in relationships such as the LSER with descriptors that will make the relationships more generally applicable. The Theoretical Linear Solvation Energy Relationship (TLSER) is such a derivation, using the LSER philosophy and general structure, but replacing the empirically derived descriptors with computationally derived descriptors.

2. Calculational Procedures

All geometries were optimized using the MNDO algorithm within MOPAC.[6,7] The molecular volumes were generated from the optimized geometries using the method of Hopfinger, as incorporated in the U.S. Army developed molecular modeling package MMADS.[8,9] All experimental data and LSER parameters were taken from works of the original authors.

3. The TLSER Descriptors

The TLSER descriptors have been developed with two main goals in mind. First, the TLSER descriptors should correlate optimally with the LSER parameters.

Second, the property correlation equations with the TLSER should yield correlation coefficients and standard deviations as accurate as the LSER. Further, the TLSER descriptors should be as generally applicable to any solute/solvent interaction as are the LSER descriptors.

The TLSER descriptors that have been developed follow that of the LSER, and fit into one of the categories listed in equation 1. The steric term for the TLSER is the molecular Van der Waal's volume (V_{mc}). Volume calculations of this type are standard in most molecular modeling packages available today. As would be expected, correlation between V_m and V_{mc} is high, with a correlation coefficient of 0.979 and a standard error of the estimate of 5 ml/mol.

The polarizability term used in the TLSER is determined from the method of Stewart and Dewar incorporated in MOPAC[10]. Dividing the resulting polarizability by the V_{mc} results in a size independent Polarizability Index (π_I). This term defines the eases in which the electron cloud can be moved or polarized. Aromatics would rank high on the scale, and alkanes low. In addition, the π_I is inversely proportional to the electronegativity.

Like the LSER, the hydrogen bonding term is separated into acid (acceptor) and and base (donor) terms. Furthermore, because the hydrogen bond (or the Lewis bond or in essence any bond) can be separated into covalent and electrostatic parts, individual descriptors are needed for the TLSER to describe this. The energies of the highest occupied molecular orbital (representing the basicity, (ϵ_b) and the lowest unoccupied molecular orbital (representing the acidity, ϵ_a) are used to describe the covalent interactions. Similarly, the electrostatic portions of the basicity and acidity are represented by selected atomic formal charges in the molecule, either the most negative (for the basicity, q_-) or the most positive hydrogen (for the acidity, q_+). The basicity terms (ϵ_I and q_-) correlate highly with the LSER β term, with a correlation coefficient of 0.9518 and a standard error of the estimate of 0.09.

The final generalized TLSER equation, is shown below:

$$\text{LOG P} = mV_{mc} + p\pi_I + a\epsilon_a + a'qH_+ + b\epsilon_b + b'q_-$$

The relationship is similar in appearance to the LSER, and contains all of the key points that have made the LSER successful.

4. Representative Correlations

Similar to the LSER, all six TLSER descriptors are seldom required or are statistically significant for a given solute/solvent property. Generally, the equations reduce to a three or four descriptor correlation. Table 1 shows a representative set of correlations using the TLSER descriptors.[11-16] In each case, the descriptors that were significant at the 95% confidence level were included, and all others dropped from the correlations. An in depth treatment of this technique is given in a recent journal article, and several technical reports.[17,18] The next paper will describe some correlations completed in depth pertaining to selected physical properties.

Table 1. TLSER Correlations

Property	V_-	π_1	ϵ_s	ϵ_s	q_-	qH_+	C	N	R	SD
Kow	2.995	-0.847	1.730	n/a	-5.415	n/a	-3.960	64	0.957	0.357
LC_{50}	-0.928	-10.557	-1.442	n/a	0.443	n/a	18.082	32	0.943	0.574
EC_{50}	-4.067	-4.147	3.902	n/a	-2.777	n/a	11.356	25	0.982	0.332
EA	n/a	-2444	n/a	3540	1430	-383	20545	23	0.951	308
k_m	1.185	-0.610	n/a	n/a	-2.290	-1.077	-0.083	19	0.986	0.073
k_{ox}	-97.7	874	1256	n/a	n/a	n/a	-2184	10	0.986	26.9
C(Tadpole)	-2.13	-4.891	-4.980	n/a	5.00	n/a	11.99	39	0.979	0.237
C(Gold Orfe)	2-2.64	94.585	n/a	n/a	4.15	-1.77	7.55	30	0.975	0.237

5. Conclusions

The diversity of the properties successfully correlated with the TLSER show the general applicability of both the LSER and the application of theoretical techniques to the LSER. In addition, the TLSER descriptors, based solely on theoretically derived and determined parameters, result in as good a correlation as the LSER descriptors. Using these equations, then, solute/solvent interactions can be related to fundamental descriptions (structural and electronic) of the molecule. Furthermore, using the TLSER descriptors, *a priori* prediction of solute/solvent properties can be made using these equations.

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BIOGRAPHY

George R. Famini has been a research chemist in the Research Directorate of the Chemical Research, Development and Engineering Center since 1981. He has been active in the application of computational chemistry to problems in chemical defense. He has served as the CB Simulants Program Coordinator since 1989. He is also active in the American Chemical Society, serving on the Program Committee of the Division of Computers in Chemistry, and on the Executive Committee of the Division of Chemical Information.

EFFECTS OF PHOSGENE AND PERFLUOROISOBUTYLENE ON PERMEABILITY OF PULMONARY ENDOTHELIAL CELLS IN CULTURE

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Dose response and time course profiles of post exposure permeability were used to determine effects of edemagenic gases on barrier function of endothelial cells (EC). Dye exclusion assays indicate that phosgene doses of 1/2, 1, 2, and 5 LCt 50s increased EC permeability by 12, 17, 26 and 57% respectively. The effect was immediate, lasted for at least 9 hours and does not explain the "clinical latent phase." Permeability was unaffected by 20 minute exposures to perfluoroisobutylene (PFIB) at doses of up to 8 LCt 50. Results suggest that inhalation of phosgene and PFIB may induce different cellular mechanisms to effect pulmonary edema.

Phosgene (CG) and PFIB are toxic, low molecular weight, organohalide gases. Both are practically insoluble in water. Yet, when inhaled into the moist environment of a lung, each of these gases triggers a delayed physiological response that overwhelms the air-blood barrier and floods the alveoli with edematous fluids. It is not certain which cells and mechanisms of the airway and air-blood barrier are targets of these gases. Clearly, the first cells to be exposed during inhalation are epithelial cells lining the airways. Pawlowski and Frosolono[1] have claimed that the initial histopathology from CG occurs in the bronchioles. Diller et al.[2] have claimed that CG-induced histopathology begins at the air-blood barrier. We surmise from these studies that there is some correlation between increased dose and progressive involvement of more distal portions of the airway. However, a considerable body of evidence suggests that increased permeability following inhalation of toxic gases is a consequence of direct microvascular[3] and endothelial[4,5] injuries. These mixed results on the pathophysiology of toxic gases lead us to believe that the mechanisms effecting inhalation injury and loss of barrier function are complex and elusive subjects limited by strict adherence to studies with animal models. Cultures of pulmonary tissues provide complementary and powerful alternatives, especially for the study

of target cells and mechanisms. We have developed cell cultures for this purpose and show in the following study how endothelial models have helped to investigate EC-gas interactions and to understand their involvement in the edemagenic process.

MATERIALS AND METHODS

Endothelial cells from the distal pulmonary arteries of male sheep were grown to confluence in suspension cultures on Cytodex-3™ microcarrier beads (Pharmacia LKB, Piscataway, NJ). Media consisted of HAM's F-12 supplemented with 15% fetal bovine serum (FBS), 50 μg/ml endothelial cell growth factor, 1% L-glutamine, 50 μg/ml gentamicin (Sigma Chemical Co., St. louis, Mo.) and 5% Omni™ serum (Advanced Biotechnologies Inc., Columbia, Md.). Under optimized growth conditions, beads seeded with 30,000 cells/cm^2 reached confluence in 7-10 days and produced high density stationary phase populations in excess of 160,000 cells/cm^2. An additional 8 days of growth were required before populations developed cell-to-cell barriers tight enough to expose and test for effects of edemagenic gases on permeability. Dye exclusion assays (Fig.1) were used to determine whether permeability of EC barriers were altered by direct gas exposure. For these assays we used erythrosin-B, a red viability stain with a negative (-) charge and a peak absorbance of 526 nm. Working solutions contained 8.3 μg of stain in 1ml of phosphate buffered saline. Cytodex-3 beads pre-coated with 60 μg/cm^2 of denatured, covalently bound type-I collagen carried a net positive (+) surface charge. When 2 ml of stain were mixed for 1 minute with 80,000 beads, without cells (Fig.1, path A), roughly 93% of the stain was adsorbed onto the (+) surface of the beads and was cleared from solution as the beads settled. The remaining 7% of the stain could not be adsorbed and remained in solution. When bead surfaces were covered with confluent, untreated, control populations, the cells and their net (-) surface charge blocked adsorption and clearance of the stain (Fig.1, path B).

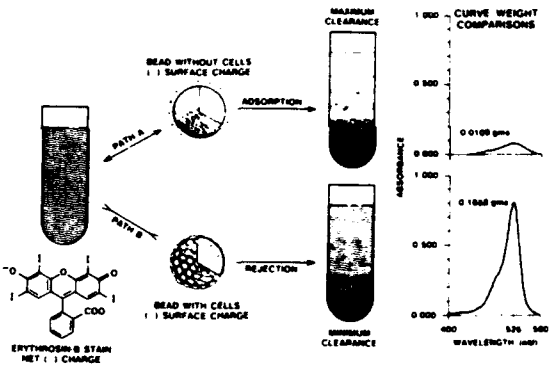

Fig. 1. DYE EXCLUSION ASSAY.

Prior to exposure, EC populations were washed 3 times in HAM's F-12 medium without serum, then split into 2 equal portions for use as controls and experimentals. In dose-response studies, samples from the experimental portion were exposed to CG or PFIB in a humidified (92% rh) gas-tight chamber. Phosgene or PFIB was injected into the chamber at dose equivalents of 1/2 to 5 LCt 50s for sheep. Each gas was chased with 150 cc of CO_2 which raised pCO_2 in the chamber to 5%, i.e., 38 mm Hg and roughly the CO_2 tension of a lung. Gas samples were allowed to equilibrate for 2 minutes before 10 μl samples were drawn for analysis by gas

chromatography. Sham-treated control populations were exposed to 5% CO_2 and air only. Because CG and PFIB are insoluble, supernatant media had to be removed from microcarrier cultures prior to exposure.

Following exposure, microcarrier populations were suspended in working solutions of erythrosin-B and subjected to dye exclusion assays as illustrated in Fig. 1 above. When the beads had settled, supernatants from these erythrosin_B solutions were scanned in an SLM Aminco DW-2C spectrophotometer. The curves generated were copied on bond paper, cut, weighed and compared with results from untreated and sham-treated controls. Curve weight comparisons were used to quantify changes in permeability of the EC barrier.

RESULTS

Dose response studies (Fig. 2) showed that 40-minute exposures to phosgene at 1/2, 2 and 5 LC 50s increased EC permeability and clearance of erythrosin-B by 12, 26 and 57% respectively.

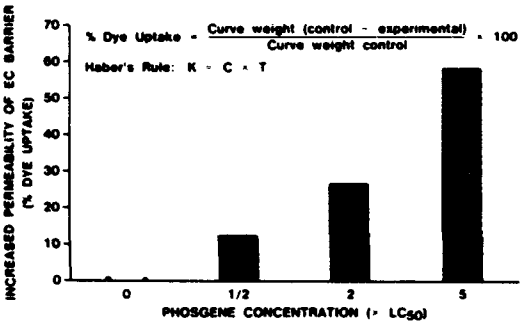

Fig. 2. DOSE RESPONSE TO PHOSGENE.

According to Haber's Rule (K = C x T), dose related increases in endothelial permeability, i.e., the biological effect (K), were the product of phosgene concentration (C) and duration of exposure (T). In all subsequent phosgene experiments, exposures were adjusted to 1 LC 50 for 20 minutes making results from *in vitro* and *in vivo* studies comparable. Post exposure viability remained >98% throughout each experiment and cells remained well attached to microcarrier beads.

Onset and duration of phosgene-induced endothelial lesions were determined in a series of post exposure permeability profiles. Results in Fig. 3 give the actual and relative amounts of erythrosin-B excluded by EC barriers of untreated controls, sham-treated controls and exposed populations. Each bar presents information obtained from 4 separate profiles run at T = 0,3,6,9 and 22 hours following sham or CG exposures. Dye exclusion by untreated control populations was consistently high and variability between assays was low. Differences between sham-treated and untreated controls were barely significant. By contrast, populations exposed to CG responded with an immediate and substantial increase in permeability. Adsorption of stain through the EC barrier of these populations remained between 17.29

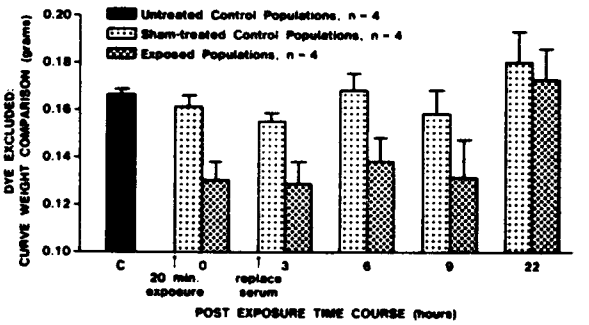

Fig. 3. PHOSGENE INDUCED PERMEABILITY PROFILE.

and 18.63% during the first 9 hours of the post exposure profile. For technical reasons, sham-treated and exposed populations were maintained in serum free medium between T = 0 to T = 3 hours. At T = 3 hours, cultures were resuspended in medium containing 15% FBS. Manipulation of the media did not alter permeability substantially; however, sham-treated and exposed populations both showed a slight, temporary improvement in dye exclusion at T = 6 hours, after serum replacement.

PFIB, unlike phosgene, did not undergo rapid hydrolysis and decay in a moist media rich environment. In fact, doses of 1 to 8 LC 50s remained stable in the exposure chamber without any detectable changes in GC analyses for periods of up to 4 hours. Under conditions identical to those used with phosgene, results presented in Fig. 4 show that PFIB at 1 LCt 50 had no effect on permeability. In fact, the EC barrier remained unaffected by 20-minute exposures to doses as high as 8 LCt 50s, and PFIB produced no delayed effects during 9- and 22-hour post exposure profile periods.

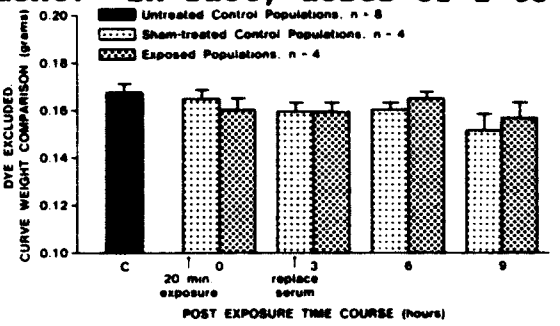

Fig. 4. PFIB INDUCED PERMEABILITY PROFILE.

DISCUSSION

CG and PFIB are lung toxicants that produce pulmonary edema. We do not know, however, which cells are targets of these gases or which mechanisms they trigger to increase permeability. With cultures of pulmonary EC and tests for permeability we were able to study endothelial lesions and answer questions that are difficult to address *in vivo*. Our results suggest that the etiology of lesions producing pulmonary edema differ for the 2 gases. This is consistent with *in vivo* results. For example, Keeler and colleagues have shown that post-exposure treatment with ibuprofen (an inhibitor of cyclooxygenase) protected rats against lethal doses of CG but was ineffective against PFIB (personal communication). There is also evidence from animal studies which support our observation that pathogenesis begins immediately after gas exposure.[6,7] However, with each of these gases, the clinical symptom, i.e., pulmonary edema, is typically delayed by a dose dependent latent phase of 3-6 hours.[2,8] The fact that exposure to CG caused an immediate increase in EC permeability *in vitro* does not explain the "clinical latent phase". It does underscore that phenomenon, however, as an expression of the lung's complex and overlapping homeostatic mechanisms. Of the 8 different cell types recognized in mammalian airways[9] each has a different structure and function. *In vitro* studies with other chemicals[10] also indicate that organ response to various chemicals can be cell type specific. It is logical to conclude, therefore, that our capacity to develop protective treatments against inhalation injuries produced by these gases and other chemicals can be enhanced by the study of their

cell specific interactions and the use of cultures as models for this purpose.

CONCLUSIONS

Phosgene causes paracellular leakage *in vitro* and increases permeability of EC barriers in a dose dependent fashion. At doses of 1 LCt 50, CG increased permeability by as much as 18.23% and produced lesions that lasted at least 9 hours. The immediate onset of increased permeability in culture does not explain the "clinical latent phase" associated with post exposure pulmonary edema. PFIB, even at doses of 8 LCt 50, did not increase permeability of EC barriers. The data suggest that use of cell cultures can provide complementary and valuable information about target cells and mechanisms by which phosgene and PFIB affect pulmonary edema.

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A Three-Dimensional Human Skin Model for Toxicity Testing

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ABSTRACT

A three-dimensional, human skin model, developed at Advanced Tissue Sciences, has recently been used as a substrate for topical toxicity testing of undiluted or high concentrations of raw materials and finished products similar to those used in human or animal skin patch tests. The skin^{2TM} Barrier Function Model consists of several layers of actively dividing, metabolically active, neonatal foreskin-derived, human fibroblasts grown on nylon mesh in the presence of ascorbate, and a basal layer of epidermal keratinocytes, several layers of differentiated keratinocytes and a stratum corneum. The substrate is placed atop a Millicell polycarbonate culture insert (3 μm pore size) and serum-free DMEM-based medium is placed below. Test articles are applied undiluted (or diluted in medium) onto the stratum corneum; the mesh is assayed for cytotoxicity using the MTT viability assay and the culture medium beneath the insert is assayed for release of PGE_2 (inflammatory mediator) and lactate dehydrogenase (membrane integrity). We have tested petrochemicals, cosmetic and personal care products and have obtained excellent correlation of the multiassay *in vitro* data with *in vivo* skin irritation data.

INTRODUCTION

Several *in vitro* alternatives to animal testing are currently being studied worldwide in order to assess the toxicity of a number of chemical compounds, raw materials and formulated products and consequently to reduce the number of research animals used. Few of these assay systems have been subjected to extensive validation studies in order to assess their intra- and interlaboratory reproducibility and relevance to *in vivo* toxicity data. A three-dimensional human skin model, developed by Advanced Tissue Sciences (skin^{2TM} ZK1300 Barrier Function Model), consists of several layers of actively dividing, metabolically active, neonatal, foreskin-derived fibroblasts grown on nylon mesh in the presence of ascorbate, and a basal layer of epidermal keratinocytes, several layers of differentiated keratinocytes and a stratum corneum. Fibroblasts growing within this three-dimensional framework secrete a number of growth factors and extracellular matrix proteins. In the current study, this substrate was utilized to study the *in vitro* toxicity of a number of compounds including petrochemicals, cosmetic and personal care products by topical application of the test materials directly to the stratum corneum surface of the epidermis. Test agent-induced toxicity was assessed by the MTT (mitochondrial function) viability assay, release of the inflammatory mediator prostaglandin E_2 (PGE_2), and release of the cytosolic enzyme lactate dehydrogenase (LDH) from cells whose membrane integrity has been compromised. *In vivo:in vitro* comparative toxicity data are very encouraging.

MATERIALS AND METHODS

Preparation Of The Barrier Function Model. **Neonatal foreskins (from routine circumcisions) were enzymatically digested with trypsin to mechanically separate the epidermis from the dermis. Further treatment yielded individual keratinocytes which were cultured in serum-free medium and expanded in monolayer. The dermis was digested with collagenase to release fibroblasts which were expanded in monolayer in DMEM supplemented with fetal bovine serum. Fibroblasts were seeded onto medical grade nylon mesh and grown for 26 days in DMEM (10% calf serum and 100 μg/ml ascorbate) to form a Dermal Model. The cells proliferate on the mesh by stretching across the interstices created by the mesh fibers. They become confluent in 2 to 3 weeks and synthesize a 3-dimensional network of collagen and extracellular matrix proteins. Keratinocytes were then seeded (1×10^5 / cm^2) onto the Dermal Model. The co-culture was grown submerged for one week, then it was lifted to the air/liquid interface for an additional three weeks. These cultures were found to have the optimal morphology and barrier function. The sheets of nylon mesh are cut with a laser (Texcel; Westfield, MA) into 11 X 11 mm squares which are used as the substrate for the topical toxicity testing described in this paper. An histological cross-section of the mesh depicts the multilayered fibroblasts, differentiated keratinocytes and stratum corneum (Figure 1).**

MTT Assay Procedure: The 11 X 11 mm Barrier Function Model ZK1300 mesh squares were removed from the MilliCells after test agent treatment, rinsed free of test article, and placed into 6-well plates containing 2 ml of 2 mg/ml MTT diluted in Serum-Free DMEM-based Assay Medium at 37°C in 5% CO_2 (≥90% humidity) for two hours. Untreated control mesh squares were also incubated with MTT. The cultures were incubated for 2 hours, then washed twice with PBS. Blue formazan precipitate was extracted from the mitochondria using 4 ml isopropanol on a shaker platform at room temperature for 1 hour. Aliquots (100 µl) of the extracted MTT solutions were diluted 1:2 with isopropanol, then transferred to 96-well plates and the optical density at 540 nm (OD_{540}) was determined using a microplate reader making a blank correction to pretreated nylon mesh (without cells) which had been similarly treated with MTT. The mean OD_{540} of the duplicate untreated control wells was set to represent 100% viability. Results are expressed as percentage of untreated control.

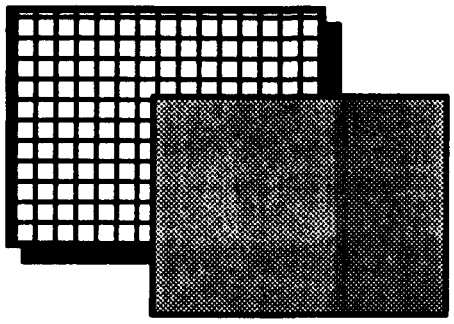
PGE_2 Assay Procedure: The Serum-Free Assay Medium under the cell cultures was assayed for test agent-induced release of cellular PGE_2, using a commercially available PGE_2 ELISA kit from Advanced Magnetics (Cambridge, MA).

LDH Release Assay Procedure: **Release of LDH from the Barrier Function Model cells was quantified using a commercially available kit from Proteins International (Rochester Hills, MI) and a standard curve using purified human LDH purchased from Sigma (St. Louis, MO).**

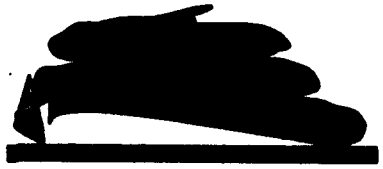
Human Skin Patch Testing: **A 14-day cumulative irritation test was performed using 20 people who received daily patches of the test substances on their backs. Results of this testing were generously provided by Dr. Thomas Stephens of In Vitro Alternatives, Inc (Carrollton, TX).**

Topical Testing Protocol

1. Tissue substrate & applicator pad

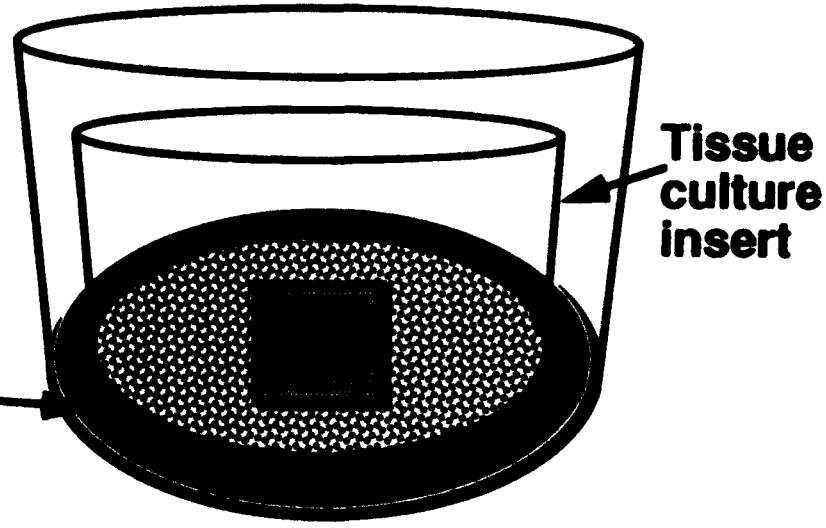


2. Test substance placed on pad



3. Pad inverted & placed on substrate

4. Pad + Substance + Substrate



Topical Testing Protocol
Barrier Function Model ZK1300

1. Barrier Function Model 11 x 11 mm tissue substrate is removed from shipping tray and placed atop MILLICELL™ culture insert.

2. Serum-free assay medium is added to chamber beneath insert.

3. 50 µl or 50 mg of test substance is applied to applicator pad, which is placed atop the stratum corneum surface of tissue substrate.

4. Incubation is for 30 minutes to overnight, depending on class of test agent.

5. Tissue substrate viability can be checked by conversion of MTT to formazan (mitochondrial activity).

6. Assay medium in lower chamber can be assayed for LDH release (membrane integrity) and PGE_2 release (inflammatory mediator).

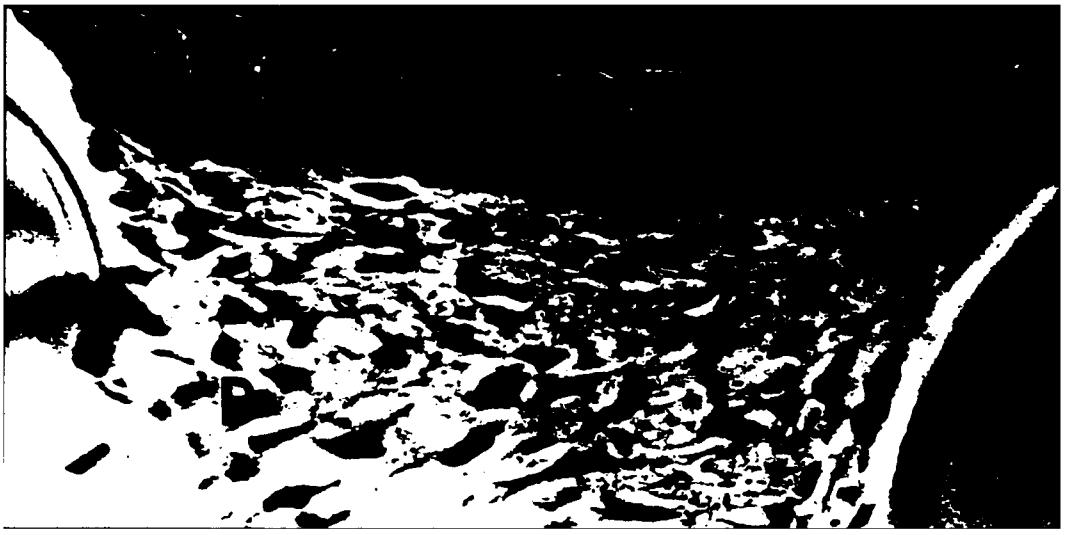


FIGURE 2

Conditioners

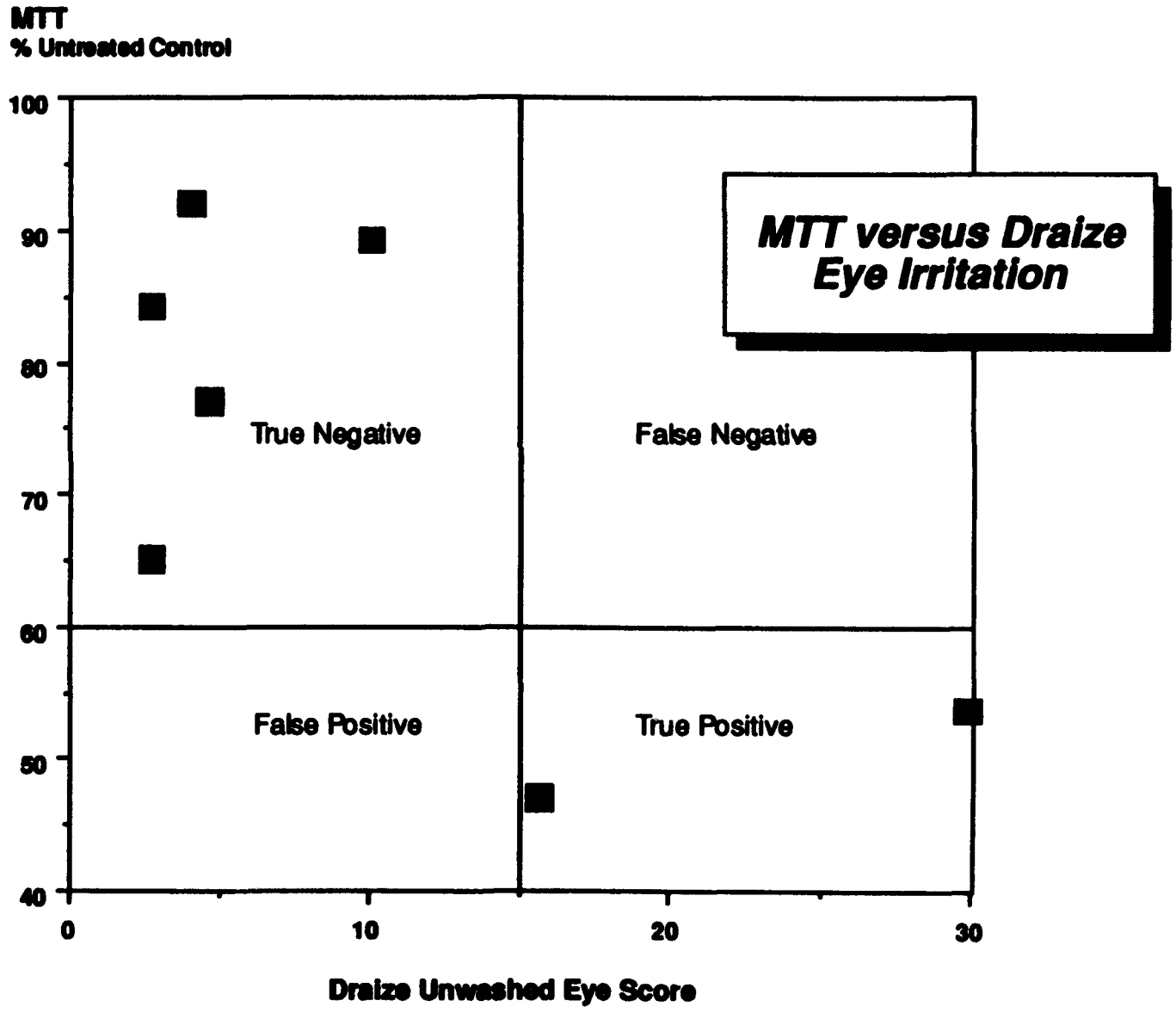


FIGURE 3

Petrochemicals

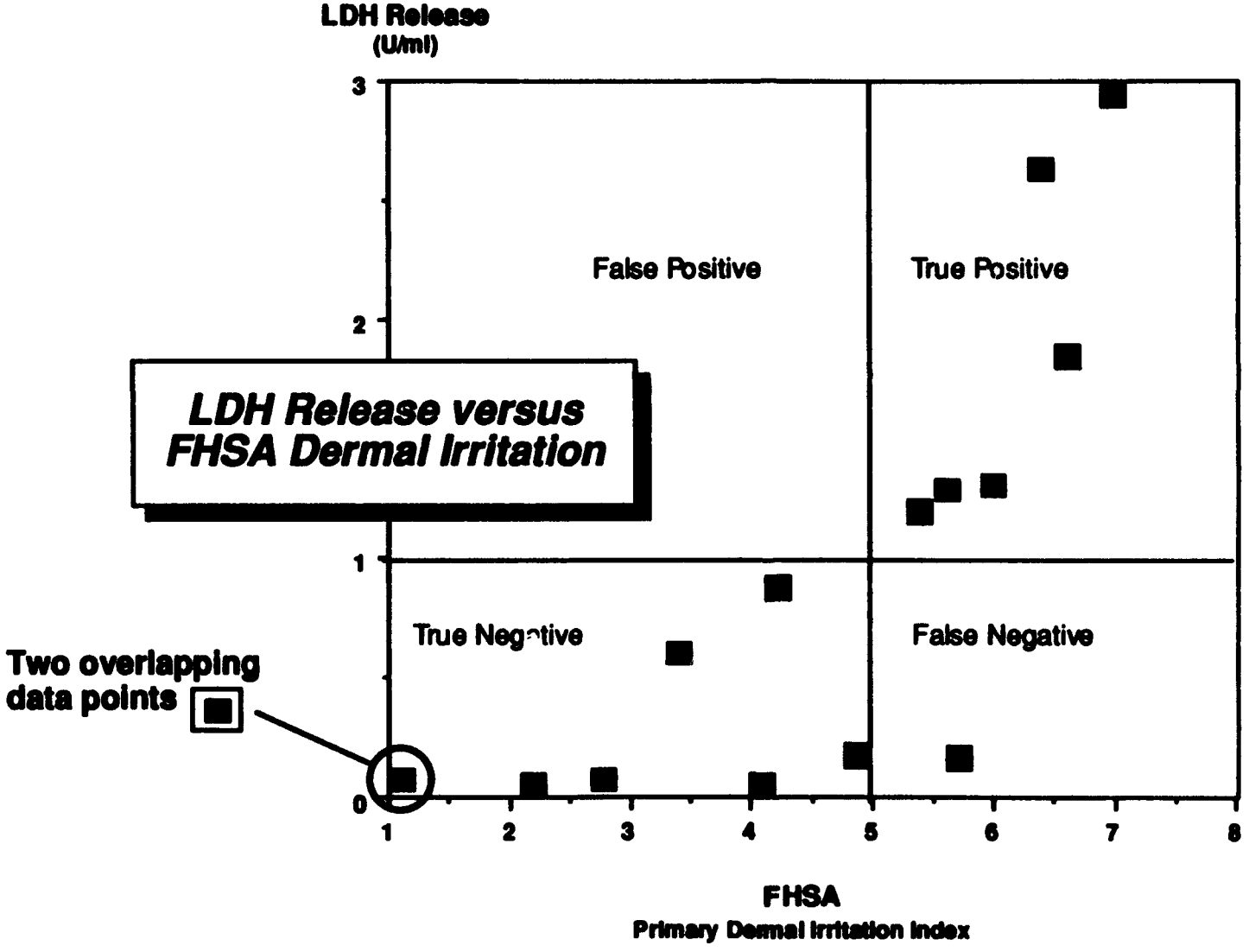


TABLE 1

Comparison of MTT and LDH Data for Cosmetic and Personal Care Products

Test Agent 2 hour exposure	MTT Assay (% Untreated Control)	LDH Assay (Units/ml)
Eye shadow	94	0.08
Blush	92	0.08
Lipstick	88	0.48
Foundation	85	0.16
Mascara	72	0.22
Hair spray	93	0.07
Deodorant	76	0.00
Face powder	91	0.13
Facial depilatory	63	2.44
Perm solution	38	2.10
Perfume #1	13	2.39
Perfume #2	30	1.41

Test Materials

TABLE 2

Comparison of Human Skin Patch Irritation Data to In Vitro PGE_2 Release

Test Materials

Test Agent 24 hour exposure	Human Skin Patch Classification	PGE_2 Release (ng/0.1 ml)
Mask	mild	1
Toner #1	less than mild	2
Sunscreen product SPF–15	mild	3
Facial cleanser	mild	5
Gel product	mild	7
Moisturizer #1	mild	8
Toner #2	mild	9
Moisturizer #2	mild	10
Lipstick	nonirritating	14
Hydroalcoholic containing botanicals	mild	179
Facial cleanser	moderate †	324

† Caused moderate acne in some subjects

CONCLUSIONS

1. We have successfully utilized the Advanced Tissue Sciences Skin^{2TM} Barrier Function Model ZK1300 in three *in vitro* alternative assay systems (MTT, PGE_2 and LDH) measuring three different mechanisms of toxicity.

2. We have obtained toxicity data for undiluted cosmetics, petrochemicals and personal care products.

3. We have shown good correlation with *in vivo* dermal and ocular irritation data for these test agents.

LEGENDS

Figure 1. Barrier Function Model Histology. Fibroblasts surrounded by naturally-secreted extracellular matrix proteins form the dermal layer (D). A proliferative basal layer, differentiated keratinocytes and stratum corneum (SC) form the epidermis (E). A nylon mesh fiber (M) is seen at right in cross section. (H & E stain, 1000X).

Figure 2. Seven conditioners were applied undiluted to the stratum corneum surface of the Barrier Function Model and incubated for one hour at 37°C/5% CO_2 in a humidified incubator. A Draize *in vivo* ocular irritation cutoff score of 15 and an MTT *in vitro* cutoff value of 60% viability correctly classified the 2 positives and 5 negatives.

Figure 3. Fifteen petrochemicals (8 lubricants, 2 organophosphate esters, 1 organic acid base condensate, 3 metalworking fluids and 1 surfactant) were applied undiluted to the Barrier Function Model and incubated for two hours. Using cutoff values of 5.0 in the FHSA primary irritation index (PII) and 1.00 Unit/ml LDH release, 6/7 positives and 8/8 negatives were correctly classified. The one false negative (a metalworking fluid) was correctly classified as a true positive in the MTT assay (8% viable).

Table 1. The MTT assay was performed directly on the BFM mesh cultures and released LDH was quantified in the underlying serum-free assay medium.

Table 2. PGE_2 release was quantified as described in Materials and Methods.

AN IN-VITRO SYSTEM FOR THE EVALUATION OF CYANIDE ION BINDING BY POTENTIAL CANDIDATE ANTIDOTES

L.T. RUSSELL, J.D. von BREDOW and J.A. VICK

Intoxication by cyanide continues to be a military and civilian problem which requires rapid therapy with effective antidotes. The testing of potential antidotes is limited to in-vivo systems which are expensive and require the use of numerous animals in order to reach a decision regarding the potential effectiveness of an antidote. Since many compounds tested as possible antidotes ultimately prove to be inactive, the potential exists for the unproductive loss of experimental animals.

A sensitive and rapid in-vitro system has been developed to monitor the rate and extent of binding of cyanide ions with potential antidotal compounds. In this system known concentrations of potassium cyanide (10^{-5} M to 10^{-3} M) in Tris buffered saline, plasma or whole blood are treated with candidate cyanide binding agents. The rate of depletion of the cyanide ion is monitored by the specific ion electrode system which has been described by Lebeda and Deshpande (ref. a). Candidate antidotes can be evaluated in whole blood, plasma or Tris buffered saline for the ability to interact with cyanide compared with the rate of binding to Co^{++} which has demonstrated anticyanide activity.

The in-vitro technique is able to determine if the action of a potential compound is: (1) through a direct interaction with cyanide, (2) through an indirect interaction with cyanide, (3) or if the compound is capable of employing both mechanisms. These in-vitro evaluations make possible the preliminary estimate of potential anti-cyanide activity without the use of traditional animal models.

MATERIALS AND METHODS

Potassium Cyanide (Fisher Scientific) is added to Tris (100 uM) buffered saline, horse plasma or heparinized dog blood to develop cyanide concentrations of 10^{-5} to 10^{-3} M.

Cyanide ion concentration is determined with a Cyanide specific ion electrode (Cyanide combination Ion Selective Electrode FK1502CN, combined with an Ion 85 analyzer, Radiometer, Copenhagen). Potential differences are displayed on a strip chart recorder (Kipp and Zonen) to monitor alterations in cyanide ion concentration.

Candidate cyanide antidotes are added to the constantly stirring cyanide solution to ensure rapid and complete interaction. Temperature is maintained at 37°C in a temperature bath monitored with a thermocouple (Physitemp) temperature probe. The pH of saline is maintained at 7.2 to 7.6 through the addition of 100 mM Tris buffer (Tris[hydroxymethyl]aminomethane, Sigma Chemical) and the pH of the plasma and whole blood are carefully monitored throughout the addition of the unknown antidote to the in-vitro system.

Formation of methemoglobin by antidotes was assessed in heparinized dog whole blood with a OSM-3 Hemoximeter (Radiometer, Copenhagen).

Test antidote reagents consist of:
Cobalt Chloride,
Sodium Nitrite,
Hydroxylamine HCl,
Dimethylaminophenol,
o-ethylquinolinium chloride,
ethyl 4-(4-methoxyphenyl)-2,4-dioxobutyrate, and
3,3'-tetrathiobis-N-acetyl-L-alanine.

RESULTS

The rate of the direct interaction of cobalt chloride with cyanide can be calculated from a plot of the disappearance of cyanide ion vs. log time in seconds. The plot revealed two distinct rates of interaction:

initial rate = 1.344×10^{-4} moles/sec
second rate = 6.413×10^{-5} moles/sec

The rates may be expressed in a more useful manner which states that 25% of the total cyanide is bound in 1.0 second
and 73% of the total cyanide is bound in 10.0 seconds.

Of all of the compounds tested only Cobalt Chloride demonstrated a direct interaction with the cyanide ion,

The following compounds do not interact directly with cyanide ion in solution

Sodium Nitrite
o-ethylquinolinium Cl
Hydroxylamine HCl,
Dimethylaminophenol
3,3'-Tetrathiobis-N-acetyl-L-alanine
Ethyl 4-(4-methoxyphenyl)-2,4-dioxobutyrate

The following compounds form methemoglobin and interact indirectly with cyanide ion in solution

Sodium Nitrite
Hydroxylamine HCl
Dimethylaminophenol

In heparinized dog whole blood the rate of interaction of cyanide and methemoglobin is similar to the rate of interaction of cyanide and cobalt ion.

DISCUSSION

The specific ion electrode system is limited in sensitivity to the range of 10^{-5} M to 10^{-1} M in buffered saline and plasma and is limited to 10^{-4} M to 10^{-1} M in heparinized whole blood. Therefore the range of anide ion concentrations and the concentration of antidotes must be maintained within an optimal range. Control of the pH with Tris buffer in saline is important since the cyanide will volatilize in an acid media leading to the disappearance of cyanide at a rate similar to the cyanide binding patterns. This phenomena can be demonstrated with the well known cyanide antidotes Hydroxylamine hydrochloride and dimethylaminophenone in unbuffered saline. Both of these compounds rapidly reduce the pH of the test solution leading to a disappearance of cyanide and an apparent interaction directly with cyanide. The same study carried out in Tris buffered saline demonstrates no apparent interaction between these compounds and cyanide. The loss of cyanide is less apparent in plasma or whole blood samples which moderate the reduction in pH caused by these compounds.

This is an aqueous system with a limited capability to interact with water insoluble compounds. This limitation may be circumvented to some extent by adding up to 50% methanol to the buffered saline. The cobalt chloride standard will interact with the cyanide ion in a manner similar to the complete aqueous system. Candidate antidotes (3,3'-Tetrathiobis-N-acetyl-L-alanine and Ethyl 4-(4-methoxyphenyl)-2,4-dioxobutyrate) were dissolved in 10% methanol and added to the cyanide solution.

This in-vitro system responds directly to the added compound or to the indirect effect of the compound through methemoglobin formation. The potent methemoglobin formers, hydroxylamine hydrochloride, dimethylaminophenone and to a lesser extent sodium nitrite, demonstrate significant activity in this in-vitro model. The system does not form metabolites and candidate antidotes such as the para aminopropriophenones, which must be metabolized in-vivo to the active methemoglobin forming metabolite, would not be expected to demonstrate activity in an in-vitro whole blood assay system.

The electrode system may become less sensitive to compounds dissolved in blood or plasma (ref. b,c,d), never-the-less this electrode system responds to a change in cyanide ion concentration induced by complexation with potential antidotes which is sufficient to determine the active nature of a potential antidote.

CONCLUSION

A rapid and sensitive in-vitro assay system has been developed which is capable of defining direct and indirect interactions of potential antidotes with cyanide ion in saline, plasma or whole blood.

This in-vitro assay system may provide a useful preliminary evaluation of candidate compounds to prioritize and limit the total number of animal tests required to select successful antidotes

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MUNITIONS CYTOTOXICITIES IN VITRO

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ABSTRACT

Cytotoxicities of various environmentally important munitions and related compounds were compared by neutral red uptake assays in continuous rat liver hepatoma cells. The toxicity order for 2,4,6-trinitrotoluene associated nitroaromatic compounds was 1,3,5-trinitrobenzene>2,4,6-trinitrotoluene>1,3-dinitrobenzene≥2,4- and 2,6-dinitrotoluenes and was in general agreement with that of other systems used for munitions screening. Cytotoxic responses were also measurable for monoamines that can be formed from these compounds in the environment. Most of the amino nitroaromatic compounds were similar in their cytotoxicities and most were less toxic than the nitroaromatic compounds from which they are derived. Neither triazine and tetrazocine munitions nor their acetylated congeners elicited responses in the assay.

INTRODUCTION

Rapid, inexpensive, and reliable methods are needed for the toxicological screening of chemical substances with the potential to affect health and the environment. One such method, the neutral red (NR) cytotoxicity assay, is based on incorporation of the supravital dye neutral red into lysosomes of viable cells. The NR uptake assay can be used to detect cytotoxic/cytostatic effects of chemical substances capable of damaging cells, has been adapted to microtiter tissue culture systems, and can be analyzed by means of automated spectrophotometric microplate readers (1,2). NR cytotoxicity assays in continuous rat hepatoma H4IIE cells have been applied to a variety of environmentally important munitions and related compounds. H4IIE cells maintain inducible oxidative microsomal enzymes and were chosen because of their application to detecting other xenobiotics in environmental and biological specimens.

MATERIALS AND METHODS

H4IIE rat hepatoma cells were obtained from the American Type Culture Collection, Rockville, MD and were grown at 37°C in a 5% CO_2 atmosphere. Medium was Eagle's minimal essential medium with Earle's salts supplemented with 10% foetal bovine serum, 10% newborn calf serum, 10 mM non-essential amino acids, and 50 μg/mL gentamycin. NR uptake assays were conducted in the same medium except that gentamycin was raised to 250 μg/mL to accommodate non-sterile munitions. Dimethyl sulfoxide (DMSO) was the solvent for all test chemicals and was maintained at 0.25% (v/v) in NR uptake assays.

NR Cytotoxicity Assays (2):
Day 1. Monolayers were trypsinized, dispersed, and 96-well microtiter plates were seeded at 9 x 10^3 cells per well in 0.2 mL fresh medium.
Day 2. Medium was aspirated, and 0.2 mL fresh medium containing the desired test chemical concentration was added to each of 8 replicate wells for each toxicant concentration.
Day 3. Medium was aspirated from all wells exposed to chemicals and solvent controls and replaced with 0.2 mL medium containing NR (0.005%, w/v) for 4 hr at 37°C. NR staining solution was removed and cells were washed 2 min with a fixative (1% $CaCl_2$, 1% formaldehyde). After aspiration of the fixative solution, dye remaining in the cells was extracted with 0.2 mL 1% acetic acid, 10% butanol, and 50% ethanol for 1.25 hr. Microtiter plates were agitated 2 min to distribute the dye and optical absorbance measurements were made by means of an automatic microtiter plate reader.

Measurements: Values of NR uptake in multiple wells for each toxicant dilution were plotted as mean percentages ($\pm 1\sigma$) of controls exposed only to DMSO. Midpoint cytotoxicity values (NR_{50}) are defined as that amount of toxicant which would reduce NR uptake to 50% of its control value based on regression curves of the uptake data in the region of change.

Munitions Abbreviations: 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), 1,3-dinitrobenzene (1,3-DNB), 2-amino-4-nitrotoluene (2A-4-NT), 4-amino-2-nitrotoluene (4A-2-NT), 2-amino-6-

nitrotoluene (2A-6-NT), 1-amino-3-nitrobenzene (1A-3-NB), 3,5-dinitroaniline (3,5-DiNA), 2,4,6-trinitrotoluene (2,4,6-TNT), 1,3,5-trinitrobenzene (1,3,5-TNB), 2-amino-4,6-dinitrotoluene (2A-4,6-DNT), 4-amino-2,6-dinitrotoluene (4A-2,6-DNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 1-acetylhexahydro-3,5-dinitro-1,3,5-triazine (TAX), and 1-acetyloctahydro-3,5,7-trinitro-1,3,5,7-tetrazocine (SEX).

RESULTS AND DISCUSSION

2,4,6-TNT and Corresponding Monoamines: From plots of NR uptake versus toxicant concentrations, the midpoint cytotoxicity value for 2,4,6-TNT was determined to be 0.033 mM. The corresponding amines, 2A-4,6-DNT and 4A-2,6-DNT, demonstrated similar cytotoxicities to each other (NR_{50} = 0.449 and 0.373 mM) but were at least 10 times less toxic than the parent nitroaromatic compound.

2,4- and 2,6-DNT and Their Monoamines: From similar plots, 2,4-DNT (NR_{50} = 0.439 mM) was at least 10 times less cytotoxic than 2,4,6-TNT in the assay. Midpoint cytotoxicities for 4A-2-NT and 2A-4-NT (NR_{50} = 0.269 and 0.328 mM) were only slightly lower than that of the 2,4-DNT parent compound. Likewise, 2,6-DNT (NR_{50} = 0.384 mM) was also significantly less toxic than 2,4,6-TNT in the assay. Midpoint cytotoxicity for the corresponding amine, 2A-6-NT (NR_{50} = 0.502 mM), occurred at a concentration only slightly higher than that of the 2,6-DNT parent.

1,3-DNB, 1,3,5-TNB and Their Monoamines: Plots of NR uptake demonstrated that the most potent compound studied was 1,3,5-TNB (NR_{50} = 0.0099 mM). It was more than 3 times as cytotoxic as 2,4,6-TNT. A microbial metabolite of the compound, 3,5-DiNA (NR_{50} = 0.132 mM) showed considerably reduced midpoint cytotoxicity. A second nitrated benzene compound found in munitions wastestreams, 1,3-DNB (NR_{50} = 0.298) was far less toxic than 1,3,5-TNB in the assay. Its amine 1A-3-NB (NR_{50} = 1.1 mM) demonstrated considerably reduced cytotoxicity as compared to the parent compound.

Triazines and Tetrazocines: RDX and HMX and their acetylated derivatives, SEX and TAX, showed no significant cytotoxicity at any concentration after three separate assays for each compound. The highest concentrations applied to the system (100 mg/L) far exceeded the aqueous solubilities of the compounds.

Comparative Cytotoxicities with Two Other Screening Systems: As is shown in table 1 for TNT and related nitroaromatic compounds, relative potencies in NR uptake assays are in the order TNB>TNT>DNB$\geq$DNTs. Except for 2,6-DNT which was somewhat more toxic than 1,3-DNB and 2,4-DNT in water fleas, toxicity orders are in general agreement. Correlation coefficients between NR_{50} values and LC_{50} values were 0.83 for water fleas and 0.91 for fathead minnows. For the corresponding amines in the table, with the exception of 3,5-DiNA and 1A-3-NB, NR_{50} values clustered from 0.27 to 0.5 mM with overlapping ranges and direct correlation with LC_{50} values is not seen. Most monoamines were less toxic than the parent nitroaromatics in NR

uptake and were less toxic than the parent nitroaromatics in NR uptake and fathead minnow assays, whereas the opposite was true with water fleas. None of the systems correlates with acute rat oral toxicities for which the order is DNB>>DNTs>TNB>TNT, although the monoamines 2A-4-NT, 1A-3-NB, 2A-4,6-DNT are also less toxic in rats than corresponding nitro compounds. No significant cytotoxicity was noted for triazine and tetrazocine associated munitions in the assay. Although data is sparse, there appears to be little toxicity associated with RDX for Ceriodaphnia dubia and other invertebrates and with HMX for fish up to the limits of their solubilities (5). In contrast, acute toxicity has been reported for RDX in fathead minnows (4).

TABLE 1

NR_{50} values for munitions and related compounds in H4IIE cells and their acute toxicities to water fleas and fathead minnows.[a]

	H4IIE			Water flea	Fathead minnow
COMPOUNDS	NR_{50} (mM)	(-1σ to +1σ) (mM)	r	48 hr LC_{50} (mM)	96 hr LC_{50} (mM)
2,4,6-TNT Associated Nitroaromatics					
1,3,5-TNB	0.0099	(0.0083-0.012)	0.99	0.013 0.014[b]	0.0052 0.0024[b]
2,4,6-TNT	0.033	(0.023-0.044)	0.99	0.052	0.013
1,3-DNB	0.298	(0.247-0.354)	0.98	0.295 0.163[b]	0.042 0.1[b]
2,6-DNT	0.384	(0.316-0.453)	0.91	0.12	0.102
2,4-DNT	0.439	(0.408-0.472)	0.97	0.261	0.18
2,4,6-TNT Associated Amino Nitroaromatics					
3,5-DiNA	0.132	(0.101-0.164)	0.99	0.084 0.075[b]	0.12 0.116[b]
4A-2-NT	0.269	(0.183-0.381)	0.98	0.09	0.16
2A-4-NT	0.328	(0.204-0.480)	0.98	0.15	0.45
4A-2,6-DNT	0.373	(0.298-0.458)	0.97	0.027	0.035
2A-4,6-DNT	0.449	(0.354-0.547)	0.96	0.023	0.077
2A-6-NT	0.502	(0.381-0.640)	0.96	0.093	0.33
1A-3-NB	1.1	(1.0 -1.202)	0.96	-	-
Triazines and Tetrazocines					
RDX	none			none[c]	4-6 mg/L[d]
HMX	none			-	none[d]
SEX	none			-	-
TAX	none			-	-

a. Data from Liu et al., 1984 (3) unless otherwise noted.
b. Data from van der Schalie, 1983 (6).
c. Data from Peters et al., 1991 (4).
d. Data from Rosenblatt et al., 1991 (5).

CONCLUSIONS

All 2,4,6-TNT associated munitions tested and their monoamines have responded measurably in NR uptake assays in H4IIE cells. Triazine and tetrazocine munitions, RDX and HMX, did not respond, nor did their acetylated congeners SEX and TAX. Responses to 2,4,6-TNT and associated nitroaromatic compounds were essentially consistent with two other systems used to screen munition samples, while the monoamines they form in the environment responded differently. Coupled to compatible extraction methods, NR uptake assays are potentially valuable in detecting 2,4,6-TNT and associated compounds or their mixtures in contaminated samples, whether or not they have been reduced in the environment. The same is not true of RDX and HMX associated munitions.

DISCLAIMER

The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other official documentation.

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IN VITRO METHODS FOR HEPATOTOXIC ASSESSMENT OF HALOGENATED FATTY ACIDS

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Running Head:
Hepatotoxic Assessment In Vitro

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SUMMARY

The use of *in vitro* toxicity screens can give insight into mechanisms of chemical toxicity. Halogenated hydrocarbons comprise an important class of compounds used by the Air Force and industry. Recently, the relative *in vivo* rank order of toxicity of three selective halogenated hydrocarbon acids was C10-perfluoro-n-decanoic acid > C8-chlorotrifluoroethane carboxylic acid > C6-chlorotrifluoroethane carboxylic acid. *In vitro* biochemical and fluorescence assays of various hepatocyte species functions were used to correlate previous *in vivo* findings. The results of this study correlated well with that seen *in vivo* and demonstrates the usefulness of *in vitro* techniques to reduce animal use and cost.

INTRODUCTION

The John Hopkins center for Alternatives for Animal Testing has estimated that approximately 17 to 22 million animals are used annually in the US laboratories, and that testing of a new chemical would cost ~1.5 million dollars. Furthermore, pressures from animal rights groups will result in a need for alternatives to whole animal testing. Total replacement of whole animal testing is not realistic at this time. However, animal reduction and refinement are achievable. It has been recently reported that animal use has been reduced by the employment of *in vitro* methods (1).

Halogenated hydrocarbons comprise an important class of chemicals used by the U.S. Air Force and industry. These compounds have been found to possess physical properties that are suited for meeting various Air Force needs. Some of these compounds are ideal candidate hydraulic fluids, lubricants and extinguishants due to their low flammability. However, these compounds have been shown to be both nephrotoxic and hepatotoxic (2,3). Furthermore, these compounds have been shown to induce hepatic peroxisome proliferation (4,5) which has been linked to tumorigenesis in rodents (6).

Many qualitative and quantitative methods exist for measuring cellular toxicity *in vitro*. Endpoints of toxicity for selected target cells can range from cell death (7) to short term reversible alterations in cellular structure and function (8). Biological potency can be correlated with chemical structure and ranked by employing cells in bioassay screens. The rational for

the use of cultured cells to assess toxicity is based on the premise that "the actions of chemicals that produce disease and death in animals are ultimately exerted at the cellular level" (9), and is reviewed in detail by Bradlaw (10).

The purpose of this study was to determine if *in vitro* methods could be utilized to screen halogenated fatty acids for hepatotoxicity and peroxisomal fatty acid ß-oxidation induction. Utilizing *in vitro* biochemical and fluorescent assays, critical cellular and subcellular changes can be identified that are not easily assessed *in vivo* and can give insight into the development of hypotheses of mechanisms of toxicity. This will result in a reduction of animals and cost in the evaluation of chemical toxicity.

MATERIALS AND METHODS

Rat Liver Perfusion

Fischer 344 rat livers were perfused and hepatocytes were isolated and enriched as previously described (11) with the following modifications: 1) Perfusion media were supplemented with 15 mM HEPES. 2) Washout perfusion medium was supplemented with 0.5 mM EGTA. 3) Digestion medium was supplemented with 0.26 U/mL of collagenase (Type A; Lot CGB- 118913, Boehringer-Mannheim Biochemicals, Indianapolis, IN).

Primate Liver Perfusion

An aseptic recirculating perfusion system was designed to perfuse pieces of Rhesus monkey (Macaca mulatta) liver. An approximate 10 gm liver biopsy section was cut into 3 pieces (~3.0 gm). Digestion medium was the same as that used to perfuse rat livers, except that Sigma (St. Louis, MO) collagenase Type IV (Lot # 109F6804) was used at 317 U/mL. Liver pieces were digested for 45 min.

Hepatocyte Isolation and Culture

Primary rat and primate hepatocytes were isolated by low speed isodensity Percoll (Pharmacia, Piscataway, NJ) centrifugation as described by Kreamer et al. (12). Rat hepatocytes (2×10^8 cells) and primate hepatocytes (4×10^7 cells) were adjusted to a cell density of 1×10^6 cell/mL, respectively. Two milliliters of cell suspension was added to rat tail collagen pre-coated 60 mm Falcon tissue culture plates (0.2 mg/plate). After attachment, rat hepatocytes were refed with Williams E culture medium (Gibco, Grand Island, NY) supplemented with HEPES (18 mM), sodium bicarbonate (2.2 mg/mL), bovine serum albumin (0.5 mg/mL), 5-aminoluvelinic acid (0.1 mg/mL), Sigma insulin/transferrin/sodium selenite solution (10 μg/mL), gentamycin (50 μg/mL) and 5% fetal calf serum (FBS; Gibco, Grand Island, NY). Primate hepatocytes were refed with Williams E culture medium supplemented as above, except that 5% Stimucyte serum-free media supplement (Upstate Biotechnology, Inc., Lake Placid, NY) was used as a substitute for serum.

Exposure

Sterile stock solutions of trimer CTFE acid, tetramer CTFE acid and PFDA were made-up in DMSO at concentrations of 10 mg/mL, respectively. The final concentration of test agents and DMSO in tissue culture plates were 50 μg/mL (0.1 mM) and 0.5%, respectively. Plates were dosed daily with change of culture medium. Rat hepatocytes were exposed for 96 h while primate hepatocytes were exposed for 120 h. At the termination of exposure, hepatocytes were scraped using a rubber policeman in 150 μL of 154 mM KCl/50 mM Tris-HCl buffer (pH 7.2-7.4). Hepatocyte samples were then homogenized by sonication on ice.

Biochemical Assays

Medium lactate dehydrogenase (LDH) enzyme levels were determined using a DuPont ACA V discrete clinical analyzer (DuPont, Huffman Estates, IL) as previously described (11). Acyl CoA oxidase (ACO; 13) and carnitine acetyltransferase (CAT; 14) peroxisomal enzyme activities were assayed as previously described. Cytochrome P_{450} activity, as determined by 7-ethoxycoumarin-o- deethylase (7-ECOD) activity, was assayed according to the method of Gray et al., (13). Reduced glutathione (GSH) was assayed according to the method of Hissin and Hilf (15) as described by Tyson and Green (16). Glutathione-S-transferase (GST) was assayed according to the method of Ahmed et al. (17). Protein determinations were assayed according to the method of Bradford (18).

Fluorescence Plate Reader

Dose response studies were conducted in twenty four-well Corning plates (Fisher Scientific, Pittsburg, PA) that were seeded at approximately 2 x 105 cells/well. After 24 h, cells were dosed at four dose levels ranging from 25 (0.05 mM) to 500 µg/ml (2.0 mM) for in 0.2 ml with tetramer CTFE carboxylic acid and PFDA and incubated an additional 24 h , respectively. Following exposure, cell monolayers were washed with calcium free-phosphate buffered saline (CF-PBS).

Rhodamine-123 (R-123; 1.0 mg/ml PBS) was added to triplicate wells for each treatment group at final concentrations of 10 µg/ml. After 60 min of incubation at 37°C, the wells were washed three times in CF-PBS and 0.2 ml of CF-PBS was added to all wells. Negative controls consisted of empty wells that were treated with R-123, washed three times, and refed CF-PBS. Fluorescence determinations were quantified on the Millipore CytoFluor 2300 fluorescence plate reader using a 485nm excitation/530nm emission filter set combination at a sensitivity of 3. Data expressed as percent control.

Flow Cytometer

Primary rat hepatocytes were exposed to a low (0.2 mM) and high (2.0 mM) concentration of tetramer CTFE acid for 4 h in suspension and then analyzed by flow cytometry. Rat hepatocytes from control and treatment groups were suspended in Dulbecco's phosphate buffered saline containing 5% serum to a density of 1 x 106 cells/ml. One ml of each sample was transferred into a 5 ml

cell culture tube and 10 µl of a 100 µl/ml stock solution of R-123 added. The tubes were placed into a 37°C incubator with 5% CO_2 atmosphere for 30 minutes. Immediately following the incubation the samples were run on a FACScan (Becton Dickinson) flow cytometer, using an argon laser for excitation at 488nm. Light was collected from the forward scatter (FSC), side scatter (SSC) and fluorescence 1 (FL1, 530/30nm) detectors. Cell populations were displayed as dot plots of FSC vs. SSC. Debris was identified and excluded by establishing a gate to include intact cells and gated cells then were displayed as FSC vs. FL1. Untreated cells established the control values; subsequent samples were compared to this control in the Fl1 parameter.

Statistics

Biochemical measurements for each treatment group were compared by an analysis of variance (ANOVA) using SYSTAT software statistics package (SYSTAT, Inc, Evanston, IL). Means found to be significant by ANOVA were compared with the Tukey post hoc test with Type I error level held at $p < 0.05$.

RESULTS

Rat hepatocytes and primate hepatocytes were exposed to PFDA, trimer CTFE acid and tetramer CTFE acid (Fig 1) at a dose of ~0.1 mM for 96 and 120 h, respectively. The dose chosen (0.1 mM) was based on previous in vitro toxicity studies with PFDA using primary rat hepatocytes. This dose of PFDA was found to result in severe toxicity in hepatocytes and was used for

comparative purposes with the CTFE acids at similar doses.

Exposure of rat or primate hepatocytes to PFDA resulted in significant cytotoxicity (Fig 2). Primate hepatocytes exposed to PFDA in serum-free medium resulted in significant cell loss over the exposure period such that levels of LDH could not be detected. Rat hepatocytes exposed to PFDA in medium containing 5% serum resulted in a significant 4-fold increase in LDH leakage when compared to control cells (Fig 2). This 4-fold increased LDH leakage represented 40 ±10% of the total available LDH present in control cells. Control cell LDH leakage was found to be 10% of total LDH.

Only primate hepatocytes exposed to the tetramer CTFE acid resulted in a significant 8-fold increase in LDH leakage when compared to control (Fig 2). This 8-fold increase represented 27 ± 3% of the total LDH. Control LDH leakage from primate hepatocytes was ~3% of the total. Exposure of rat hepatocytes to either trimer or tetramer CTFE acids did not result in any significant change in LDH leakage when compared to control (Fig 2).

Exposure of rat hepatocytes for 24 h to either the tetramer CTFE acid or PFDA over a dose range of 0.05 to 1 mM indicated similar dose-response effects as determined by mitochondrial R-123 fluorescence measured in a fluorescence plate reader (Fig 3). A 300% increase in R-123 fluorescence occurred at the highest dose of PFDA (0.4) and tetramer CTFE acid (1 mM) used. No dose-response effects were found for the trimer CTFE acid in rat hepatocytes exposed at concentrations ranging from 0.2 to 1.4 mM

(data not shown). Rat hepatocytes were exposed to a low (0.2 mM) and high (2.0 mM) concentration of tetramer CTFE acid for 4 h and then analyzed for R-123 fluorescence using a flow cytometer. Flow cytometric analysis indicated a significant 71% reduction in R-123 fluorescence at the high CTFE acid concentration when compared to control and low CTFE acid concentration (Fig 4).

Rat and primate hepatocytes were exposed to the halogenated carboxylic acid test agents at 0.1 mM for 96 and 120 h, respectively, and the activity of peroxisomal ACO and CAT enzymes were then determined. Control ACO activity in rat and primate hepatocytes could not be determined under the experimental conditions used. However, ACO activity was measurable in rat hepatocytes exposed to the test agents (Table 1). In contrast, no ACO activity could be determined in similarly exposed primate hepatocytes. Rat hepatocytes exposed to the trimer and tetramer CTFE acids indicated significant 8-fold and 7-fold increases in CAT activity when compared to control, respectively (Table 1). A significant 2-fold increase in CAT activity was only observed in primate hepatocytes exposed to the trimer CTFE acid.

Hepatocyte metabolic and detoxification capabilities were assessed in 96 and 120 h exposure cultures. Cytochrome P450 activity, as determined by 7-ECOD activity, was only found to be significantly increased in rat hepatocytes exposed to the trimer CTFE acid (Table 2). Measurements of 7-ECOD activity in primate hepatocytes were not determined. Exposure of rat hepatocytes to either PFDA or tetramer CTFE acid resulted in significantly reduced GSH levels (Table 2). However, GST activity was not

significantly reduced (Table 2). Exposure of primate hepatocytes to the tetramer CTFE acid resulted in significant reductions in GSH level and GST activity (Table 2). Due to cell loss from the culture plates, GSH level in primate hepatocytes exposed to PFDA could not be measured by the method employed here. While GST activity was found to be significantly reduced in these cells, it can not be attributed to treatment because the cell number in the culture plates was reduced.

CONCLUSIONS

Halogenated hydrocarbons comprise an important class of compounds used commercially and by the U.S. Air Force. These compounds have been found to possess physical properties that make them ideal candidates for hydraulic fluids, lubricants and fire extinguishants. The principle property of these compounds that make them attractive is their low flammability.

Previous *in vivo* work with PFDA indicated a 14 day lethal dose 50 (LD50) of ~64 mg/kg in male Fischer 344 (F-344) rats (19). The 14 day LD50s for the tetramer and trimer CTFE acids were recently found to be ~88 and ~396 mg/kg in male F-344 rats, respectively (20). In the present *in vitro* study PFDA was found to be the most toxic at 0.1 mM based on LDH leakage and cell loss from culture plates in both rat and primate hepatocytes. The tetramer CTFE acid only induced significant LDH leakage in primate hepatocytes exposed under serum-free culture conditions. However, this LDH leakage was not as great as that in PFDA exposed rat hepatocyte cultures containing 5% serum. Exposure of

either rat or primate hepatocytes to the tetramer CTFE acid also resulted in significantly reduced GSH levels. A number of studies have shown a correlation between GSH depletion or GST inhibition and toxicity or carcinogenicity (17,21,-24). No significant effect on LDH leakage or GSH levels was observed in either rat or primate hepatocytes exposed to the trimer CTFE acid. Therefore, these data indicate that PFDA was the more toxic than the tetramer CTFE acid which was more toxic than the trimer CTFE acid. These *in vitro* results correlate well with the previously established *in vivo* LD50s for these compounds.

The greater LDH leakage observed in primate hepatocytes exposed to the tetramer CTFE acid is more than likely due to the low amount of protein in the StimuCYTE™ supplemented serum-free culture medium. StimuCYTE™ is a low-protein culture medium supplement that can be used as an equivalent to serum to support the growth of a number of cell types (25). This medium supplement was used to prevent fibroblastic overgrowth. The reduced levels of protein in these cultures may allow for a higher percentage of free tetramer CTFE acid that can interact with cellular membranes or proteins resulting in greater LDH leakage.

Interestingly, only the trimer CTFE acid was found to significantly induce cytochrome P^{450} activity in rat hepatocytes as determined by 7-ECOD activity. The significance of this induction with respect to toxicity is not known. It is possible that the trimer CTFE carboxylic acid is effectively neutralized by the induction of a particular cytochrome P_{450} isozyme that is

not inducible by longer chain halogenated carboxylic acids. Additional *in vitro* studies using sensitive fluorescent probes for different isozymes of cytochrome P_{450} and parallel metabolic *in vivo* studies are required to help elucidate the involvement of cytochrome P_{450} in determining halogenated fatty acid induced hepatotoxicity.

The ability of PFDA and "parent" tetramer CTFE to induce peroxisomal proliferation and ACO activity have been previously demonstrated in the rat (4,26). In contrast to the previous *in vivo* exposure of rats to the "parent" tetramer CTFE oligomer, exposure of rhesus monkeys to this CTFE oligomer did not result in any increase in peroxisomal ACO activity (27). Recently, exposure to the tetramer CTFE carboxylic acid, but not the trimer CTFE carboxylic acid, has been shown to result in increased peroxisomal ACO activity in the rat (28).

In the present *in vitro* study, rat hepatocytes exposed to all three test agents demonstrated measurable ACO activity, while identically exposed primate hepatocytes did not. This species difference is in agreement with the previous *in vivo* studies described above. The increase peroxisomal ß-oxidation observed in primary rat hepatocytes exposed to the trimer CTFE carboxylic acid is in contrast to that observed *in vivo* (28). An *in vitro* system does not allow for chemical or metabolite elimination that can occur *in vivo* in exposed animals. Therefore, chemicals or metabolites may interact with hepatocytes in vitro where they would not *in vivo*.

The increase in CAT activity in rat hepatocytes exposed to

the experimental halogenated fatty acids is not surprising since it has been shown that this activity is co-induced with ACO with exposure to other classes of compounds (13). A significant increase in CAT activity was also observed in primate hepatocytes exposed to the trimer CTFE carboxylic acids. The significance of this increased activity in primate hepatocytes is not known. However, it does not appear that increased CAT activity in primate hepatocytes would be associated with toxicity or carcinogenicity because other associated changes in the liver (hepatomegaly) do not occur as they do in the rat (27). As of yet, it is not clear what the relationship between peroxisomal ß-oxidation induction and carcinogenicity is.

The results from this study indicate that primary hepatocytes are a useful and inexpensive research tool for screening potential peroxisome proliferating chemicals, and for rank-order screening of chemical toxicity using *in vitro* biochemical and fluorescence assays. With the rapid development of novel halogenated hydrocarbon fluids for military and commercial use, it would be advantageous to develop *in vitro* systems that are inexpensive and reduce animal use to screen this class of compounds for toxicity.

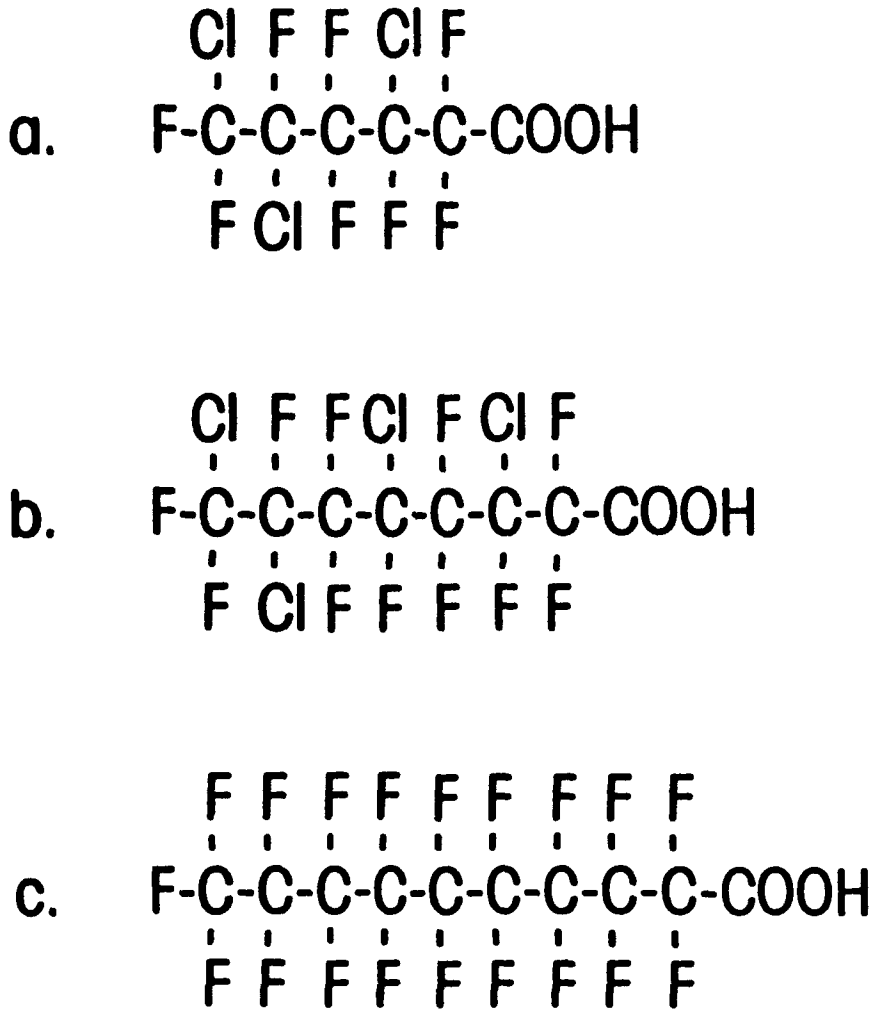

Figure I. Chemical structure of halogenated carboxylic acid test agents. a) trimer CTFE acid; b) tetramer CTFE acid; c) PFDA.

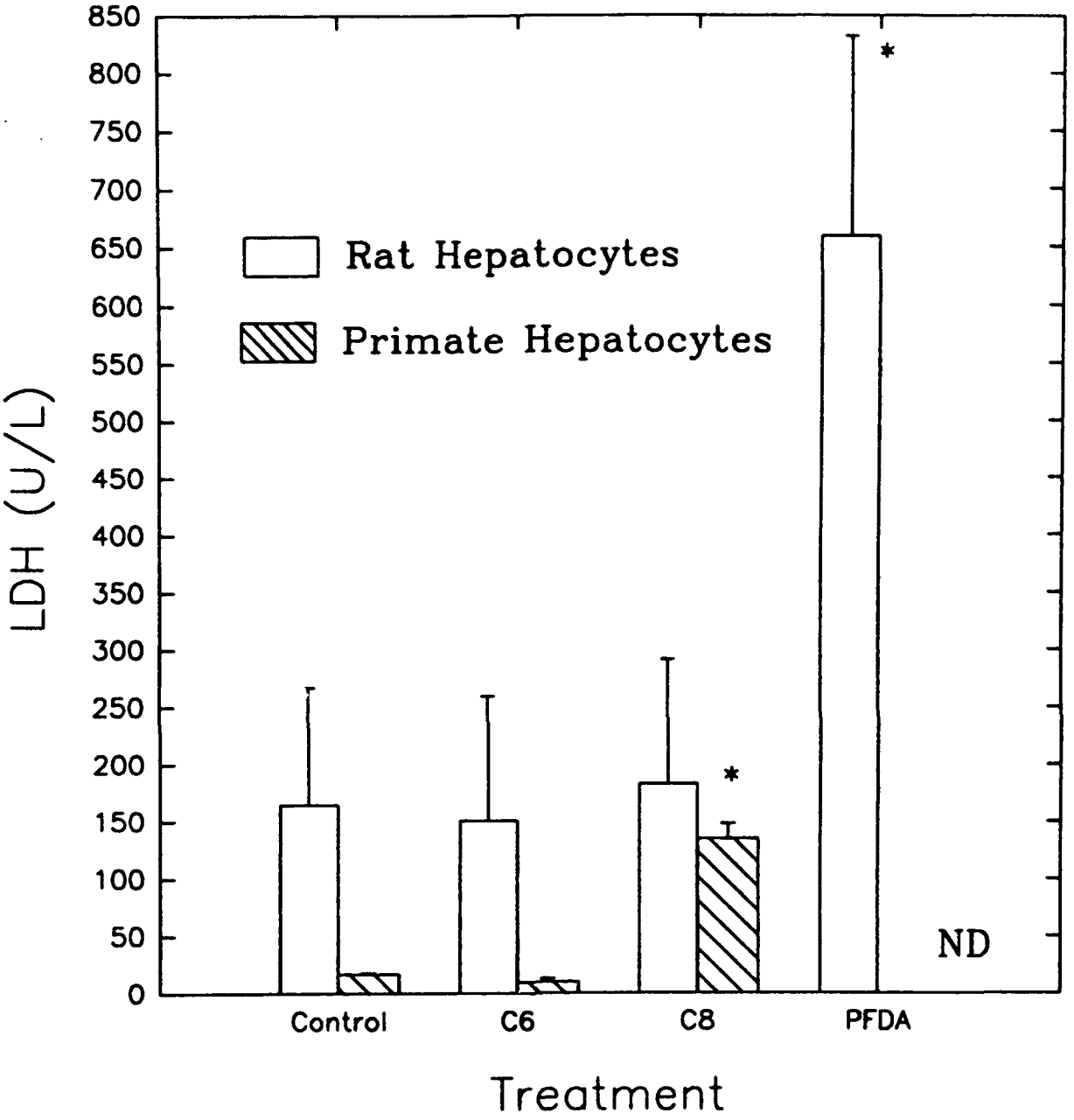

Figure II. Medium LDH enzyme levels from rat and primate hepatocyte cultures following exposure to various halogenated fatty acids at 0.1 mM for 96 h and 120 h, respectively. Bars represent the mean LDH (International units/L) from three experiments with triplicate plates per treatment group. Control LDH leakage from rat and primate hepatocyte cultures were ~10% and ~3% of total, respectively. (*) Significantly different than control at $p < 0.05$.

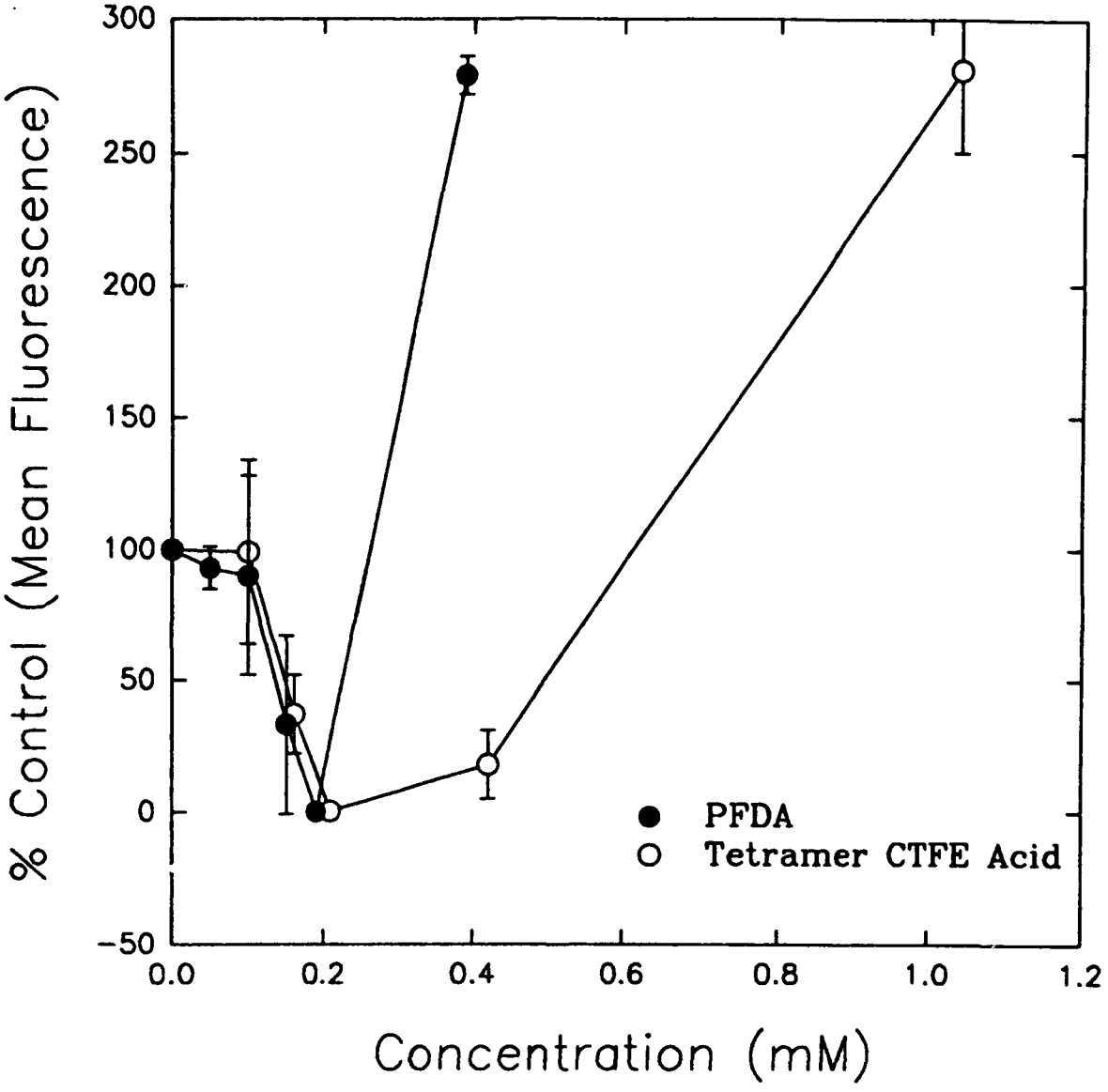

Figure III. Dose response effects of increasing tetramer CTFE acid and PFDA concentrations on rhodamine-123 dye retention by primary rat hepatocytes exposed for 24 h. Data points represent the average fluorescence from a single experiment with quadruplicate wells per treatment group. Fluorescence was determined in a CytoFluor 2300 fluorescence plate reader. The amount of fluorescence plotted is the percent of non-exposed control cells.

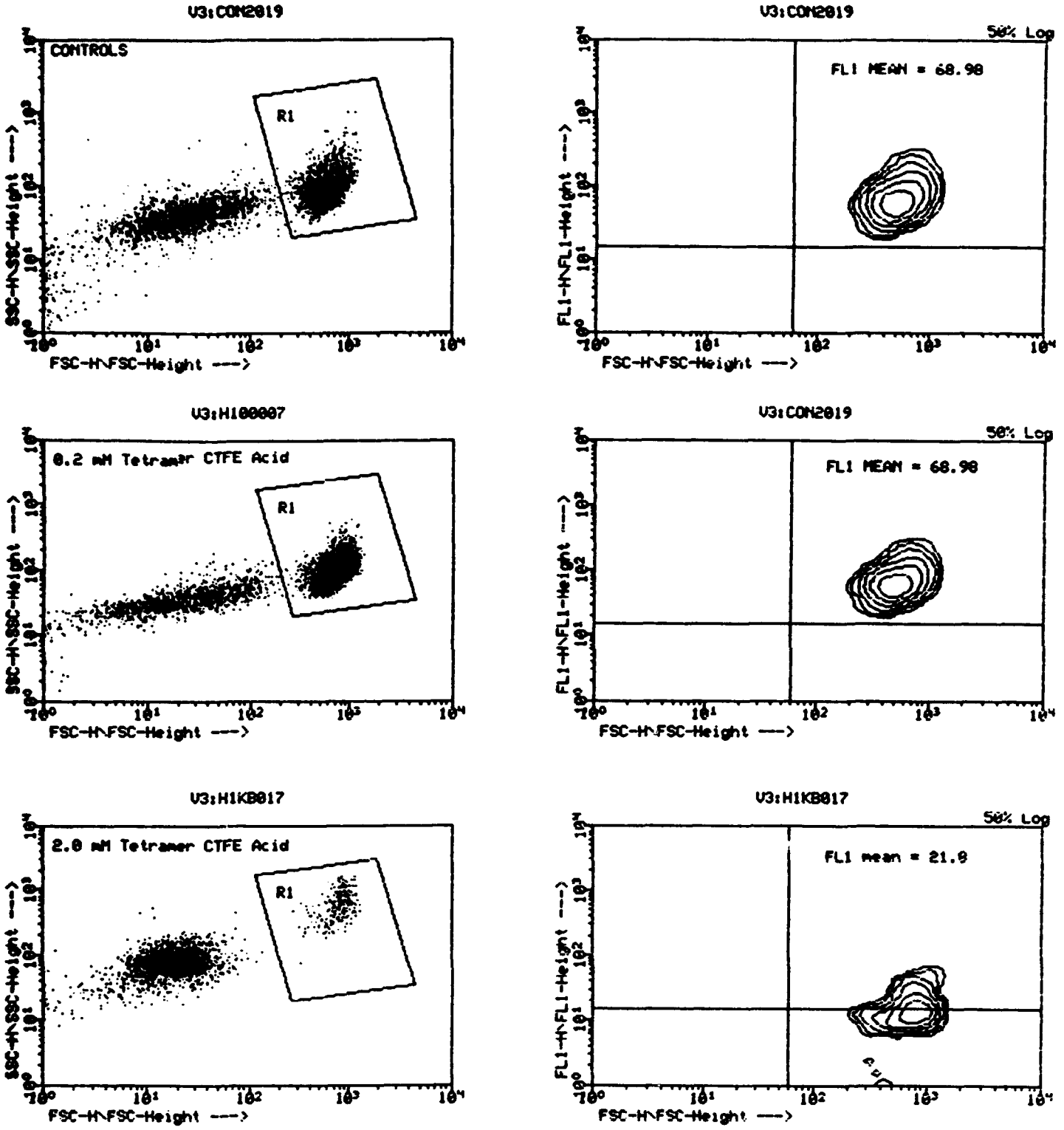

Figure IV. The effect of tetramer CTFE acid on primary rat hepatocyte size (forward scatter [FSC]; x-axis) vs granularity (side scatter [SSC]; y-axis) after 4 h of exposure in suspension culture. Contour plots on right represent intact hepatocytes (gate R1 of scatter plot). FL1 represents mean R-123 fluorescence. (*) Significantly different than control at $p < 0.05$.

TABLE 1

PEROXISOMAL FATTY ACID β-OXIDATION ACTIVITIES IN PRIMARY RAT AND PRIMATE HEPATOCYTES AFTER EXPOSURE TO VARIOUS HALOGENATED FATTY ACIDS

		Assay	
Treatment[a]	Species[b]	Palmitoyl CoA β-Oxidation (nmol/min/mg)	Carnitine Acetyltransferase (nmol/min/mg)
Control	Rat	0	3.5 ±1.4[c]
	Primate	0	5.4 ±1.3[d]
C_6 CTFE Acid	Rat	13.2 ±7.7	28.7 ±13.6*
	Primate	0	12.6 ±1.3*
C_8 CTFE Acid	Rat	11.3 ±5.3	23.7 ±11.1*
	Primate	0	7.2 ±3.3
PFDA	Rat	2.8 ±2.5	11.4 ±3.9
	Primate	0	0

[a] All treatment groups were dosed at 50 μg/mL (~0.1 mM) and 0.5% DMSO. Controls were were dosed with DMSO at 0.5%.

[b] Primary rat hepatocytes were exposed for 96 h. Primary Rhesus monkey hepatocytes were exposed for 120 h.

[c] Values represent the mean and standard deviation from 3 experiments with triplicate plates for each treatment group.

[d] Values represent the mean and standard deviation from 1 experiment with triplicate plates for each treatment group.

* Significantly different than control as determined by a multifactorial ANOVA with Tukey post hoc comparisons of significant means at $p \leq 0.05$.

TABLE 2

PHASE I AND PHASE II METABOLIC REACTION DETERMINATIONS IN PRIMARY RAT AND PRIMATE HEPATOCYTES AFTER EXPOSURE TO VARIOUS HALOGENATED FATTY ACIDS

		Treatment[a]			
Assay	Species[b]	Con	C_6 CTFE Acid	C_8 CTFE Acid	PFDA
7-ECOD (nmol/min/mg)	Rat[c]	0.25 ±0.04	0.52 ±0.11*	0.34 ±0.15	0.10 ±0.03
	Primate[d]	ND[e]	ND	ND	ND
GSH (nmol/mg)	Rat	15.7 ±3.8	22.9 ±5.0	6.0 ±0.7*	2.3 ±1.5*
	Primate	8.0 ±0.7	8.7 ±0.9	2.0 ±0.5*	NM[f]
GST (nmol/min/mg)	Rat	609.0 ±204.3	676.7 ±114.2	539.2 ±81.5	462.3 ±133.3
	Primate	689.6 ±34.7	653.2 ±119.8	248.3 ±42.0*	89.7 ±14.3*

[a] All treatment groups were dosed at 50 μg/mL and 0.5% DMSO Controls were dosed with DMSO at 0.5%.

[b] Primary rat hepatocytes were exposed for 96 h. Primary Rhesus monkey hepatocytes were exposed for 120 h.

[c] Values represent typical average means from 3 experiments with triplicate plates for each treatment group.

[d] Values represent the mean and standard deviation from 1 experiment with triplicate plates for each treatment group.

[e] ND = Not determined.

[f] NM = Not Measurable

* Significantly different than control as determined by a multifactorial ANOVA with Tukey post hoc comparisons of significant means at $p \leq 0.05$.

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NEUROBLASTOMA-GLIOMA CELLS AS A SYSTEM FOR STUDYING DRUG NEUROTOXICITY

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The neurotoxicity of prescribed and abused drugs has been traditionally explored using biochemical and behavioral testing in animals. These studies have revealed that psychostimulants, such as amphetamine and cocaine, profoundly affect both the immature and adult central nervous system. Since these drugs are capable of interactions with many neurotransmitters and cell types in the brain, our goal was to explore the mechanisms of these drugs' toxicity in a simplified neuronal system, the NG108-15 cell line, that contains features of both a mature and developing monoamine system. Changes in cell number, [^{3}H]thymidine incorporation and fine structural morphology were measured. The relative toxicity of these drugs *in vitro* paralleled the potency in existing animal models of neurotoxicity.

In vitro cell cultures and neuronal preparations have been used with much success in the field of neurobiology to elucidate the organizing structure of complex neuronal networks that form the basis of central nervous system function. The formation of such networks are established through chemically regulated communications between neuronal elements. These interactions result in rapid and flexible changes in neuronal structure that are vulnerable to a wide range of environmental influences. Many types of drugs can affect development by modulating chemical signalling between neurons at their synapses. In the prenatal, as well as the early postnatal period, these modifications of cellular communication can profoundly alter the final form of the adult brain and

ultimately modify brain function. The use of cell culture facilitates the study of nascent neuronal structure, by allowing the experimenter to carefully regulate the chemical milieu of the neuronal elements and to simplify their chemical associations by reducing the number and types of neuronal elements. Our studies have used a culture of neuroblastoma-glioma cells, NG108-15, which possesses many characteristics which enable us to model the *in vivo* effects of drugs on the brain[1]. This immortal cell line incorporates some of the characteristics of a developing neuronal system with rapid proliferation and neurite formation as well as the establishment of a monolayer plexus of mature differentiated cells with a stable expression of many neuronal characteristics[2]. The neurochemical profile of these cells includes components of a monoamine neurotransmitter system[3], which makes them an excellent model to study neuronal-drug interactions since many drugs have their effects through this neurochemical substrate [4]. In our experiments, we chronically exposed a populations of NG108-15 cells to various concentrations of drugs and examined them for changes in morphology and function. Specifically, we evaluated their viability with trypan blue exclusion, cell proliferation curves with [^{3}H]thymidine and structural changes with light and transmission electron microscopy. The importance of the resulting data is that it could (1) provide information as to how these drugs affect cellular structure in general and how a compound interacts with specific intracellular components in the brain, (2) help determine an estimate of a drug's neurotoxicity or its effects on the functional integrity of the cells in culture, and (3) give possible implications for acute or long-term consequence of a drug on the developing nervous system.

The drugs we utilized in our studies were psychostimulants, i.e., amphetamine and cocaine. These compounds are highly addictive, with their primary sites of action in the brain[5] and pose a significant concern for their effects on the developing human nervous system due to their abuse by some pregnant women[6]. While the behaviorally addictive aspects are well documented, there is not a clear understanding of the cellular mechanism of their actions or the consequence of their long term effects on the central nervous system. These drugs have been shown to bind to several sites in brain, including monoamine uptake transporters[5], and can alter the levels of neurotransmitters such as norepinephrine, dopamine and serotonin[7]. Both cocaine and amphetamine derivatives have a known toxicity associated with acute and chronic exposure in adult[8] and neonatal humans[6] as well as in animals[7,9]. However, very few studies have examined the changes that these drugs can induce on the fine structure of neural tissue[2,3]. The first part this study provides a detailed morphological description of psychostimulants effects on NG108-15 cells exposed at various times and doses.

Ultrastructural Morphology The NG108-15 cell line is well characterized in terms of it ultrastructure and biochemistry[1,2,10]. We found control cells to contain cytoplasm rich in organelles, including mitochondria, ribosomes, endoplasmic reticulum, Golgi, dense bodies, lipid droplets and virus particles. The dense bodies occurred in a variety of shapes and densities with some round and of medium density, while others were slightly less rounded and with much greater

medium density, while others were slightly less rounded and with much greater electron density. Elongated or rectangular dense bodies were also found. Electronlucent regions (probably lipid droplets) were characteristic of the densest types of bodies.

After the administration of cocaine, no cytoplasmic changes were noted, at doses up to 10^{-3} M. Some of the cells at higher doses had numerous dense bodies (Fig. 1). Morphometric analysis was not performed because the dense bodies tended to be polarized near one edge of the nucleus and not randomly distributed throughout the cytoplasm. However, nuclear responses to the cocaine were observed. After two days of exposure, nuclear invaginations were found to be filled with vesicles, and the nuclear membrane surrounding the vesicles was disrupted (Fig. 1).

Figure 1. Several large dense bodies (arrows) have accumulated near one end of the nucleus in the cytoplasm of this cell exposed to 10^{-3} M cocaine for 3 days. Within the nucleus, are two inclusion bodies (NI) consisting of numerous vesicles. 10,000 x mag. **Insert** At 2 days of exposure to 10^{-6} M cocaine, vesicles aggregated within the nucleus. A pair of membranes (large solid arrow) surrounds the vesicles, but in one area, has been disrupted and becomes a single membrane (medium sized solid arrow), and in one spot is broken altogether (arrowhead). 37,500 x mag.

Following three days of cocaine, patches of vesicles were observed, without any surrounding nuclear membrane. The vesicles ranged in size from 0.05 to 0.8 um. The larger ones tended to be clear cored, while both clear and dense cored vesicles were observed in the smaller sizes (Fig. 2).

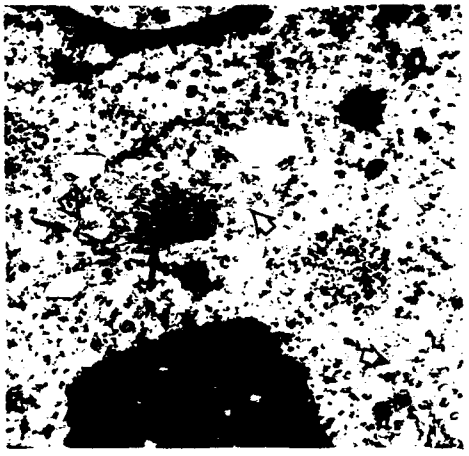


Figure 2. This cell was exposed to 10^{-6} M cocaine for 3 days. Tubular structures of different sizes (solid and hollow arrows) are present within the nucleus. It is possible that the different sized tubules are continuous with one another (arrows with asterisks). 50,000 x mag.

The contents of the nuclear vesicles varied from a fine granular matrix, to lipid-like with a dense border (Fig. 3). The tubular inclusions were 0.1 to 0.03 um in width and either round or ovoid. Vesicular patches were not, in general,

present (Fig. 3). Nuclear vesicles were not seen at one day of treatment, but at two days, vesicles appeared at the 10^{-3} M dose, and at three days, the vesicles were also present in cells exposed to 10^{-6} M cocaine. No vesiculation was seen at any time period for drug exposure lower than 10^{-6} M.

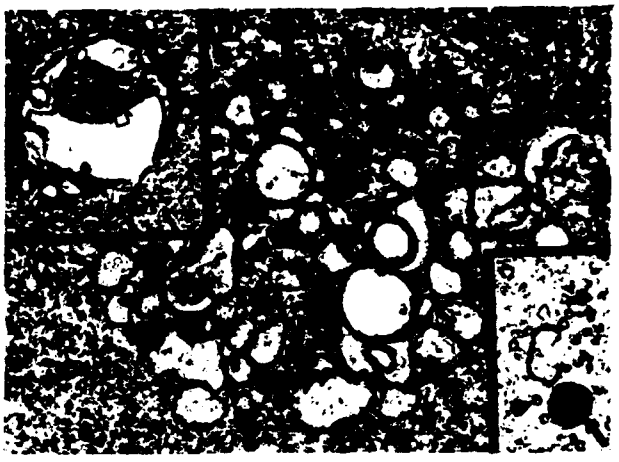
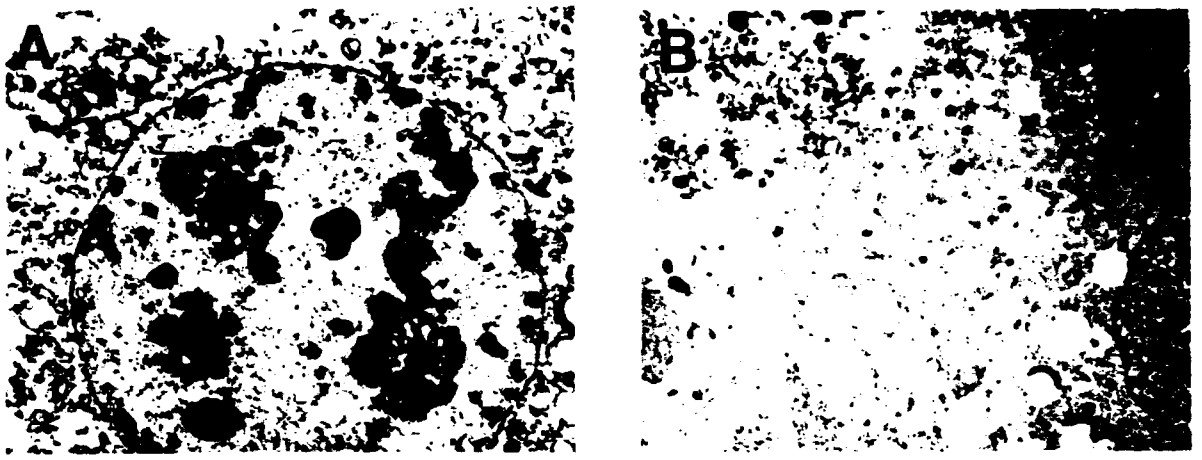


Figure 3. **(Center)** Examples of nuclear inclusions in NG108-15 cells in a culture exposed to 10^{-3} M cocaine for 3 days. Tubular structures and several membranes are shown within the nucleus. Vesicles are located nearby. 25,000 x mag. **INSERTS (Top left)** Vesicles of varying sizes, densities, and contents, are present within the nucleus. 50,000 x mag. **(Bottom right)** Numerous small vesicles (arrowhead) and one large dense vesicle or body (long arrow) were found in the nucleus. 30,000 x mag.

Increases in nuclear inclusions appeared to be the primary effect of cocaine *in vitro*. These structures may be derived from invaginations of the inner nuclear membrane and, as in the NG108-15 cells, the nuclear membrane could infold and detach to become a true inclusion. Nuclear inclusions have been found in various areas of the nervous system[11] and their number has been reported to increase following electrical stimulation[12]. Tubular structures also appear as inclusions in the nuclei of a variety of cell types. In hepatoma cells, the structures may form a direct tubular system between the nucleolus and perinuclear cisterna[13]. The tubular inclusions are also prominent in female reproductive cells, including mammotrophs of gerbils[14], human endometrium[15] and epithelial cells of patients with fibrotic lung disorders[16]. Although these intranuclear bodies have been found in a wide variety of cell types, their structural similarity suggests that their function may be similar in all cells. It appears that cells develop nuclear inclusions during periods of rapid growth or in response to specific hormonal stimuli. While the specific functional nature of the structures has not been determined; they have been hypothesized to be a series of common responses to stimulation of specific pathways of protein synthesis.

Other drugs which inhibit monoamine uptake, i.e. amphetamine-like compounds, showed some different effects at the same doses that we used with cocaine. These compounds appeared to be quite different in terms of the induced fine structural changes and cell toxicity. The exposure of NG108-15 cells to 10^{-3} imipramine (Fig. 4A) and 10^{-3} amphetamine (Fig. 4B) resulted in the disruption of cellular integrity after 3 days. The structural changes (cytotoxicity and cell death) induced by amphetamines reflected the relatively greater neurotoxic potency of these compounds seen in animal and human studies[17]. Using lower doses, we did see some nuclear changes similar to those seen in cocaine treated cells (Fig. 5).



Figures 4. Three days treatment with (A) 10^{-3} M imipramine or (B) 10^{-3} M *d*-amphetamine NG108-15 cells showed extensive degeneration characterized by vacuolated nucleoplasm and cytoplasm. 15,000 x mag.

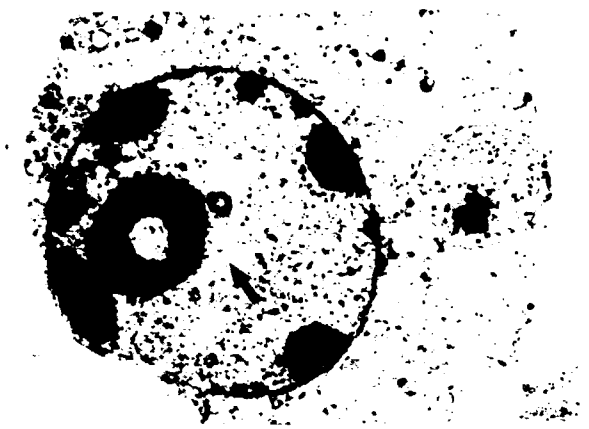


Figure 5. At 7 days of treatment with the amphetamine analog MDMA, 10^{-6} tubular nuclear inclusions were seen in this cell. 10,000 x mag.

Cell Proliferation Additional experiments were performed to measure the effect of experimental drugs on cell number and mortality. The results suggest that cocaine slows the rate of cell replication while concurrently killing a portion or perhaps select subpopulation of cells (Fig. 6).

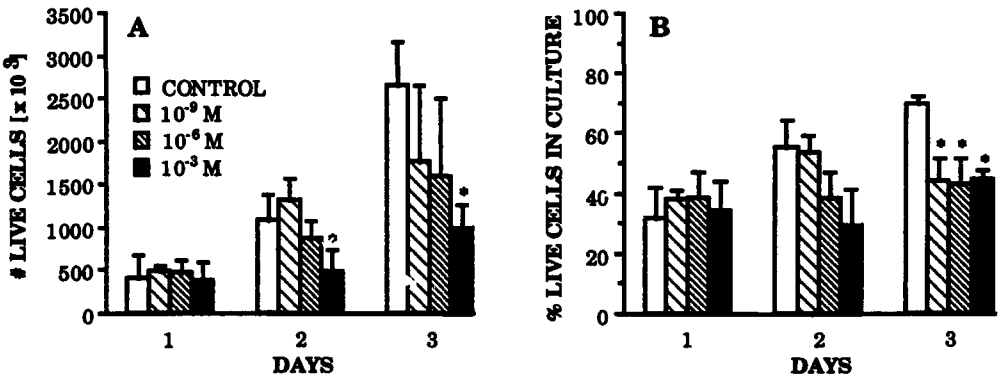


Figure 6. (A) Number of NG108-15 cells after 1,2 and 3 days of exposure to different amounts of cocaine and (B) the percent of live cells in those cultures as assessed by trypan blue staining. Asterisks indicate a significant effect of cocaine as compared to controls on the same day.

The amount of cellular division as measured by the total number of cells in culture is significantly reduced on the second day of 10^{-3} M cocaine exposure as compared to controls. Cell mortality as measured by the percent of living cells in a given culture does not appear to be significantly changed at this time and drug concentration. On the third day, a reduction in both cellular replication and survival was evident. The number of cells present in culture was still significantly depressed after exposure to 10^{-3} M cocaine. In addition, the percentage of cells that were alive in the culture, on the third day after cocaine, was significantly decreased from control for all three concentrations.

To further examine drug-induced changes in cellular proliferation, we measured DNA synthesis using [^{3}H]thymidine labelling in the cultures. This approach addressed the fine structural drug effects in the cell nucleus and the suggested differential effects on the rate of cell division and survival seen in our studies. [^{3}H]thymidine uptake increases rapidly over the first few hours as DNA is synthesized and then levels out. A dose response effect on cellular division was observed for a series of drugs, and their relative potency in inhibiting cell division agreed well with our data on cell counts and fine structure as well as their potency *in vivo*. In general, the largest impact on cellular proliferation occurred in the first 8 h after exposure to the drugs, with a maximum reduction to 20% of control values at the highest doses (Fig. 7). The inhibition of [^{3}H]thymidine uptake, by the intermediate doses, returned to control levels after 48 h of treatment with methamphetamine, but not *d*-amphetamine. There was some stimulation of [^{3}H]thymidine uptake by both drugs at the lowest levels of exposure.

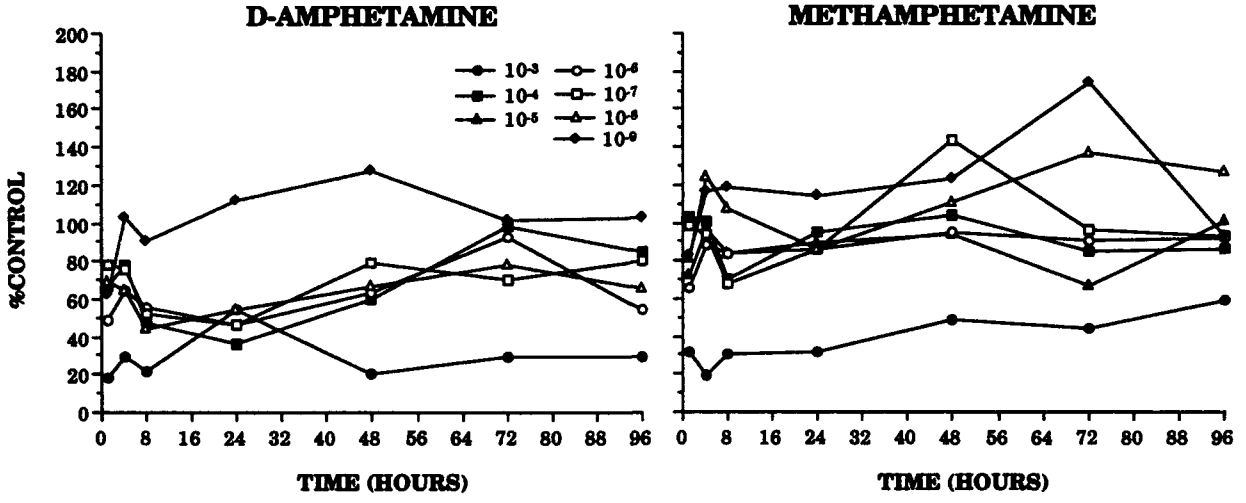


Figure 7. NG108-15 cellular proliferation over 4 days as measured by [^{3}H]thymidine uptake. Cells were grown in 96-well plates and allowed to proliferate for 12 h. [^{3}H]thymidine (5 uCi/ml was added to the medium and incubated for 30 min. Cells were then exposed to varying doses of *d*-amphetamine or methamphetamine at time zero and their growth was expressed as a percent of parallel control cultures that had no drug added.

CONCLUSIONS

These experiments have been directed at exploring the fundamental utility of using cell cultures as a medium to examine: (1) a drug's site of action at the cellular level as seen morphologically with the electron microscope; (2) the specificity of a drug's action in neurally derived cells and its relation to known neurochemical processes such as neurotransmitter functions. One primary question is whether a drug's effect, such as toxicity, is related to its interaction with an organ specific system such as the brain. Psychostimulants in our studies have major effects through the monoamine system, and their influence on morphology and cell function may be mediated through the monoamine reuptake process. Our preliminary studies suggest this to be somewhat true in that the drug's toxic potency is reduced in cells that lack this specific monoamine system or if we pharmacologically block that system. In addition, many of the psychostimulants we have used have shown a potency of neurotoxic effects that parallels that reported in a number of animal models.

The morphological data in these studies may open new avenues of research into drug effects on the developing nervous system in intact organisms. The nature of the drug-induced nuclear changes on cell growth and replication, in the cell lines suggests important structural-biochemical relationships. Our findings that cell replication is decreased and that cell mortality is increased in a time and dose-dependent manner with drug exposure may be related to the nuclear changes we observed which were also dose dependent. It seems possible that some drugs could interfere with normal replication, thus implicating the production of nuclear inclusions as a marker of genetic damage. The relation of these inclusions with chromation is presently unclear. On the other hand, cell mortality may reflect general neurotoxic effects of the drug. Our future studies will evaluate the point at which drug induced changes represent toxicity as compared to physiological, but non-toxic effects.

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THE DEVELOPMENT AND VALIDATION OF THE MINIATURE SWINE, MOUSE, AND RABBIT MODELS AS ALTERNATIVES TO THE USE OF THE DOG IN DRUG TESTING

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ABSTRACT

Because of the increasing use of propranolol and verapamil in combination, a study of their interaction was conducted. In mice, when propranolol HCl (P), was given in combination with verapamil HCL (V), death occurred within minutes and was preceded by convulsions. In rabbits, propranolol given i.v. at 1 mg/kg followed by an i.v. infusion of verapamil at 0.1 mg/kg/min for 15 minutes, caused profound hypotension, bradycardia, A-V block, and death in less than 1 hour in each of 30 animals. In minipigs, under sodium pentobarbital anaesthesia, 0.5 mg/kg propranolol and 0.5 mg/kg verapamil i.v. produced the same physiological changes and death within 15 minutes in each of 8 animals. Pretreatment of mice with calcium chloride (600 mg/kg, i.v.) prevented death due to the combination of propranolol and verapamil. In rabbits treated with propranolol and verapamil as above, 300 mg $CaCl_2$ kg given i.v. immediately after verapamil infusion restored blood pressure and cardiac conduction to normal, but the heart rate remained slow and all 12 animals survived. $CaCl_2$ given at 150 mg/kg to minipigs following the propranolol and verapamil injection prevented death of each of the 5 animals; blood pressure and the EKG were restored to normal although the bradycardia persisted. Treatment with α,β-adrenergic agonists or with glucagon was less effective in reversing the toxicity of the propranolol + verapamil combination in rabbits. The data indicate that verapamil enhances the toxicity of propranolol, and that calcium can antagonize this effect. A comparison of the data obtained in these studies with previous data indicates that the minipig and, in some instances, the mouse and the rabbit are viable alternatives to the use of the dog in studying drug interaction and therapeutic testing.

INTRODUCTION

Calcium channel blocking agents have been extensively used in Europe for the treatment of cardiomyopathies, arrhythmias and angiospastic angina (1). The use of beta blocking agents in similar cardiovascular disorders is likewise common throughout the world. It is not surprising therefore that several recent reports have dealt with the possible synergistic toxicity of the 2 drugs when used together (2, 3, 4, 5). This study was designed to investigate the possible toxic interaction of propranolol and one of the calcium channel blocking agents, verapamil, and to attempt to develop an antidote for the treatment of this type of reaction. Furthermore, these studies represent an attempt to validate the use of research animals lower on the phylogenetic scale to replace the previous use of companion animals in drug testing.

MATERIALS AND METHODS

The experiments were performed in rabbits, minipigs and mice. All studies were conducted using dl propranolol (P) and verapamil (V).

Rabbits (2.5-4.5 kg) - Control group: Each of 30 rabbits received propranolol, 1 mg/kg i.v., followed by verapamil, 0.1 mg/kg/min infused i.v. over a period of 15 minutes. Treated group: Each of 12 rabbits received propranolol and verapamil as previously described, followed by i.v. $CaCl_2$ 300 mg/kg. All rabbits were observed for 72 hours or until death.

Minipigs (24-28 kg) - Control group: 8 minipigs were given propranolol, 0.5 mg/kg i.v., followed by verapamil, 0.5 mg/kg i.v., and observed for 3 hours or until death. Treated group: 5 minipigs received propranolol and verapamil as in the control group, but were treated with $CaCl_2$ 150 mg/kg i.v. at the time at which both the heart rate and the blood pressure had decreased to 50% of control and the EKG showed signs of auricula-ventricular blockade. Blood pressure, heart rate, EKG and respiration were measured in all animals using standard recording apparatus and techniques. All animals were observed for 72 hours or until death.

Mice (20-24 gm) - Control group: 30 mice received propranolol, 13.63 mg/kg i.p., followed immediately by verapamil, 6.36 mg/kg i.p. All animals were observed for 3 hours or until death. Pretreated group: 30 mice received $CaCl_2$ 600 mg/kg i.v. prior to being given the combination of propranolol and verapamil as above. All animals were observed for 72 hours or until death.

RESULTS

Rabbits: Control group: All animals in this group died within 50 minutes after having received the propranolol and verapamil combination. Treated group: All animals treated with $CaCl_2$ survived.

Minipigs: Control group: All animals died within 20

minutes, exhibiting marked A-V blockade, profound bradycardia and severe hypotension. Respiration remained normal until shortly before cardiac arrest, at which time there was hyperpnea followed by apnea. Treated group: All animals treated with $CaCl_2$ survived. Blood pressure and heart rate decreased following propranolol and verapamil, as in the control group. Following $CaCl_2$, the blood pressure increased markedly with a significant widening of pulse pressure. Heart rate remained slow however following $CaCl_2$ and did not return to normal for at least 12-24 hours. This prolonged bradycardia did not affect the ultimate survival of the treated minipigs. The A-V block produced by propranolol and verapamil was likewise reversed by $CaCl_2$.

Mice: Control group: All animals died within 15 minutes after having received propranolol and verapamil. Pretreated group: All animals pretreated with $CaCl_2$ survived the challenge with propranolol and verapamil. The results are shown in TABLE 1.

TABLE 1

The Effect of Calcium Chloride on Propranolol-Verapamil Toxicity in Experimental Animals

Animals	Propranolol	Verapamil	Calcium Chloride	% Animal Survival
Rabbits				
Model (n=30)	1.0 mg/kg I.V.	1.5 mg/kg I.V.	---	0%
Treated (n=12)	1.0 mg/kg I.V.	1.5 mg/kg I.V.	300 mg/kg I.V.	100%
Mini Pigs				
Model (n=8)	0.5 mg/kg I.V.	0.5 mg/kg I.V.	---	0%
Treated (n=5)	0.5 mg/kg I.V.	0.5 mg/kg I.V.	150 mg/kg I.V.	100%
Mice				
Model (n=30)	13.6 mg/kg I.P.	6.4 mg/kg I.P.	---	0%
Treated (n=30)	13.6 mg/kg I.P.	6.4 mg/kg I.P.	600 mg/kg I.V.	100%

DISCUSSION

A successful model for the study of a possible toxic interaction between propranolol and verapamil was developed in rabbits, minipigs and mice. The reaction was characterized by marked hypotension, bradycardia and A-V block, representing an exaggerated pharmacological action of both drugs. Simultaneous administration of the two drugs produced death at doses well below the reported toxic range. $CaCl_2$ successfully antagonized the development of the "cardiogenic shock" in all animal models and resulted in the survival of all treated animals.

Calcium has been shown to play an important role in the excitation contraction coupling process of cardiac muscle. Depolarization of the cardiac sarcolemma is associated with an influx of calcium into the cell which initiates the release of more calcium from the sarcoplasmic reticulum to activate the myobibrils (6). Recent studies confirmed that the calcium which participates directly in myocardial contraction is derived form intracellular and extracellular sources (7, 8, 9). Therefore, it is not surprising that an ample extracellular supply of calcium is essential for normal cardiac function or to reverse the effects of calcium blockade.

Both propranolol and verapamil induce direct cardiac depression via B receptor blockade and inhibition of calcium transport, respectively, which may explain their synergistic toxic interaction (10). Administration of Ca restores extracellular-intracellular balance through the remaining patent channels, antagonizing the observed "toxic effect of propranolol and verapamil." Therefore, calcium can be considered as an appropriate antidote against the toxic interaction of beta blockers and calcium channel blockers.

Previous studies in dogs have shown a similar synergistic toxicity between the calcium antagonists and the beta blocking drugs (11). Both the decrease in blood pressure and heart rate observed in the dog appears to be similar to if not identical with the changes seen in these studies. Not only was the toxic interaction seen in the dogs quite like that observed in the minipig, but the ability of calcium to reverse these otherwise lethal effects was also the same (12).

CONCLUSIONS

Results of these studies strongly support the validation of alternative animal species lower on the animal chain to replace the dog and/or monkey in drug research.

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FEASIBILITY OF FLUORESCENCE ASSAYS IN HUMAN SKIN EQUIVALENTS WITH THE CYTOFLUOR 2300

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ABSTRACT

Biochemical and morphologic changes that may result in blister formation follow the exposure of human skin to sulfur mustard (HD, 1,1'-thiobis[2-chloroethane]) and other vesicants. Bioassay methods for screening of new antivesicant compounds should provide graded "human-like" responses that can be measured both before and after amelioration by effective antivesicants. Previous reports state that damage to human skin cell cultures is measureable by use of spectrofluorometry after incubation of fluorescent probes with such cells. Our preliminary observations suggest that damage inducible in a commercially available human skin model may be measureable with site-specific fluorescent probes.

INTRODUCTION

Antivesicant drugs are being sought for the protection of soldiers from attacks with blistering chemical agents. Therefore, vesicant damage in man must be modeled to permit measurement of damage and damage reduction during selection of safe and effective antivesicant compounds. A suitable model should be useful for the screening of such candidate compounds. Suitability for the study of the underlying mechanisms of HD is also desirable.

The hairless guinea pig model displays gross and microscopic effects of HD that are uniform, graded, and measureable.[1] However, use of animal models is to be avoided if alternative models can be used. A search for alternative methods was based on the concept that artificial human skin might substitute for animals and man. Methods for HD vapor exposures[1] were used with LSE^{R} (Living Skin Equivalent^{R}, Testskin^{R}, provided by Organogenesis, Inc., Cambridge, MA). Gross, biochemical and histological[2] signs of HD damage were observed. The development of an analytical spectrofluorometric method for observing some signs of other insults to related cells[3] was the basis for initiation of the present effort.

EXPERIMENTAL OBJECTIVE

The long-term objective of this project is to determine the feasibility of utilizing human skin models, fluorescent probes, and the CytoFluor 2300[R] instrument (Millipore Corp., Bedford, MA) to measure toxic effects induced by HD, antivesicant compounds, or both. The present effort explores concurrent use of these methods to measure damage in cells. Questions that guided this effort were:

1. Do probe/CytoFluor methods measure damage in skin substitutes?

2. Can dermal substrates bind enough of applied fluorescent probe dyes to obscure the fluorescent signals from epidermal cells?

3. Does conventional transportation damage skin substitutes?

4. Which site-specific fluorescent dyes might reveal HD damage?

APPROACHES

To address the above questions at a time when Testskin was unavailable, specimens of immature human epidermal model (HEM, 3-4 cells thick on gel-membrane) were transported to Aberdeen Proving Ground, MD (APG) from Binghamton, NY, under three different sets of conditions. Effects of transportation were checked with two dyes. When available, Testskin was used intact, or without epidermis, to address the first four questions. Eight fluorescent probes were tested as candidates for use with human epidermal models.

MATERIALS AND METHODS

A. HUMAN SKIN SUBSTITUTES GROWN IN VITRO

LSE (Testskin) and LDE[R] (Living Dermal Equivalent[R]) were furnished by Organogenesis, Inc., Cambridge, MA. Another human epidermal model was grown by using methods described below:

Millipore inserts with Millicell CM[R] microporous membranes (Millipore Corp., Bedford MA) were overlaid with a gel coating (hereafter referred to as "gel" or "gel-membrane"). The details of this technique have been patented by Millipore Corp.

Normal human epidermal keratinocytes (NHEK), as second passage cell strains and 0.025% w/v typsin/0.01% w/v EDTA solution (CC5013) were obtained from Clonetics Corporation (San Diego, CA). The NHEK were maintained in serum-free keratinocyte growth media (KGM) during growth on plastic of cell culture dishes. NHEK dissociated with CC5013 were suspended in KGM with 10% fetal calf

serum/1.5 mM calcium chloride, and seeded onto gel-membranes of inserts at a density of 3-6 x 10^5 cells/cm^2. Seeded inserts were held in media-filled wells of culture plates while cells were allowed to stratify for 5 to 7 days. The media was changed daily while the stratifying NHEK were maintained at 37°C, in 5% carbon dioxide/95% air. To achieve mature HEM, the apical medium is removed from inside the insert, exposing the apical cell surface to air for 5 to 7 days of stratum corneum formation.[4] In this effort, HEM preparations were used before they could be matured in CO_2/air.

B. FLUORESCENT PROBE ASSAYS OF HEM AND TESTSKIN

Eight different assays were accomplished with Testskin and HEM tissues. Seven assays had protocols nearly identical to those described in detail by Cook, et al.[4] Briefly, Testskin was washed three times in DMEM (Dulbecco's Modified Eagle's medium) without phenol red (GIBCO-BRL, Grand Island, NY) at 37°C. Similar washing was accomplished using HEM or gel-membrane (dermal model).

Each skin model was incubated for one hour with one of seven fluorescent dye probes or exposed to sodium fluorescein (details below). All probes are identified below by names or acronyms used by the vendor (Molecular Probes, Eugene, OR). The acetoxymethyl ester (AM) confers membrane permeability to probes identified with the -AM suffix.[5] Once inside cells, the AM is cleaved by cellular esterases to make the dye less membrane permeable.[5] The dyes and concentrations used were: calcein-AM (10 ug/ml), CMFDA (10 ug/ml), neutral red (100 ug/ml), BCECF-AM (50 ug/ml), Fluo3-AM (25 uM), rhodamine 123 (10 ug/ml) or 2',7'-dichlorofluorescin diacetate (100 uM). After incubation, each preparation was washed three more times with DMEM and read in the CytoFluor 2300, using the filter sets and gain levels listed in Table 2.

To determine dye uptake of epidermal substrates, fluorescence readings from full-thickness models were compared with readings from substrates without epidermis. Signals from LSE were compared with those from LSE after epidermis was stripped from underlying LDE with a fine forceps (subsequent to dye loading of the LSE). Signals from HEM were compared with those from gel-membrane.

Sodium fluorescein was used to assess epidermal permeability by using a different protocol from the one used with the seven fluorescent probe assays listed above. Briefly, after washing the LSE or HEM three times in DMEM, sodium fluorescein (10 ug/ml; 200ul) (Sigma Chemical Co, St. Louis, MO) was placed on the Testskin in an apical chamber. The apical chamber was made by using the silicone sealant, assay rings and directions provided with Testskin, thereby creating a well to hold dye away from the media under the Testskin. After 120 minutes (30 minutes for the HEM) the medium in the basal compartment was assayed within the CytoFluor 2300 using the filter sets and gain levels listed in Table 2. The sodium

fluorescein present in the media (basal compartment), relative to the controls, was considered to be a measure of epidermal premeability. Gel-membrane was used as the dermal control for HEM. LDE was the dermal control for LSE (LSE was not available to be stripped).

RESULTS AND DISCUSSION

A. EFFECTS OF TRANSPORTATION ON IMMATURE HEM

HEM were assayed (for damage to epidermal cells) in NY before transportation, after 5 hrs in transit (to APG, MD) followed by 18 hrs of incubation, or after 5 hrs in transit during a total time of 24 hrs without incubation. Data given in Table 1 suggest that the non-viable control (gel-membrane) may retain slightly more (100 unit changes) calcein-AM as time enroute increases. However, the viable HEM appear to leak calcein-AM at increasing rates (2000+ unit changes) with transit. The sodium fluorescein assay suggests that gel-membrane offers a slight barrier to dye leakage, but even this barrier is lost after 24 hrs enroute. Normal resistance of HEM tissue to intercellular dye leakage appears to be lost rapidly with more transportation stress. One interpretation of these observations is that travel may induce HEM cells to pull apart, one from another, while plasma membrane damage remains minimal.

TABLE 1

TRANSPORTATION EFFECTS ON PLASMA MEMBRANE AND INTERCELLULAR SPACE

Site Specificity	Dye	Control	5 hr Trip	24 hr Trip
Gel-membrane fluorescence	calcein-AM	100*	200*	300*
HEM Plasma membrane	calcein-AM	4200*	2000*	1600*
Dye leakage through gel-membrane	sodium fluorescein	4300*	4300*	6300*
Dye leakage between HEM cells	sodium fluorescein	500*	2200*	6450*

* CytoFluor 2300 fluorescence units (values rounded off). n=3.

B. OBSERVATIONS ON FLUORESCENT DYE DISTRIBUTIONS

Testskin was incubated with eight dyes of differing site specificities to see if fluorescent dye binding to dermal tissue would hinder attempts to read changes in epidermis (question 4). Table 2 shows most of the fluorescent signals from the first six dyes as coming from LSE epidermis. Similar comparisons with HEM and gel-membranes also showed most of the signal in HEM epidermis.

Table 2 shows different results with the free radical dye, and sodium fluorescein, than with the other dyes. Differences between test conditions appear to account for these differences. Dermal cells are likely to be damaged during use of forceps to strip and discard the epidermal portion of LSE. The current hypothesis is that extra dye is bound by free radicals generated in damaged dermal cells, accounting for greater fluorescence in the dermal substrates than in the whole LSE (5000 vs 1693). It remains to be seen if HD exposures act similarly. The values obtained with sodium fluorescein diverge because the signal is from dye that passes between the cells not through their plasma membranes. Table 2 shows LSE as an excellent barrier to sodium fluorescein (A,C) until epidermis is removed (B).

This effort was designed to explore the feasibility of using human skin models, fluorescent probes, and the CytoFluor 2300, in concert, to measure damage such as HD may be expected to induce. Current answers to the questions that guided experimental design are given in Table 3.

DISCUSSION OF OBSERVATIONS AND OPTIONS

It has been reported that HD vapor changes the histological profile of Testskin;[2] recent unreported observations by the same pathologists suggest that immature HEM is similarly affected by HD. Our preliminary data indicate that calcein-AM is lost from LSE and HEM after exposure to HD vapor. HEM,[4] and individual cells,[3] have shown cell-associated and site-specific changes in fluorescence after damage and incubation with various probes. Data from Table 1 suggest that immature HEM responds in the same way.

HD vapor exposures of HEM were made to obtain assurance that HD effects will be measureable by CytoFluor and fluorescent dye methods. HD exposures of HEM were made by the vapor cup method,[1] as adapted for Testskin,[2] with a 24 hr time interval between the HD challenge and incubation with calcein-AM. Transportation damage to the cultures compromised plans to compare fluorescence units with HD and controls. However, the unreported results appeared to be consistent with HD damage to plasma membranes and fluorescent dye leakage. Additional exposures are planned with dilute HD.

TABLE 2

FLUORESCENT PROBE/CYTOFLUOR 2300 ASSAYS OF LSE* AND LDE**

Parameter	Dye	A LSE +dye	B LDE +dye	C LSE only	D Net signal	E S/N ratio	F Filter set	G Gain
Plasma membrane integrity	calcein-AM	3004 ±294	1303 ±196	521 ±66	1701	3.18	485/530	4
Glutathione	CMFDA	8804 ±293	1475 ±60	103 ±13	7329	6.34	485/530	3
Lysosomes	neutral red	6540 ±186	648 ±55	116 ±12	5892	12.07	508/645	4
Intra-cellular pH	BCECF-AM	3575 ±270	1346 ±156	521 ±66	2229	3.7	485/530	4
Intra-cellular Ca++	Fluo3-AM	4685 ±327	2091 ±161	1875 ± 15	2594	13.01	500/550	5
Activity of mitochondria	rhodamine 123	5732 ±252	1494 ±228	16 ±2	2594	3.87	500/550	2
Free radicals	2,7 dichloro-fluorescein	1693 ±200	5000 ±534	103 ±13	-3307 (see text, above)	0.32	485/530	3
Epidermal permeabil.	sodium fluorescein	15 ±0	8383 ±517	14 ±0	8368 (see text, above, & #)	8369	485/530	3

* LSE (Living Skin Equivalent) and LDE (Living Dermal Equivalent) are registered trademarks of Organogenesis, Inc, Cambridge, MA.
** In this case, LSE with epidermis removed to leave LDE.
± Standard error of the mean.
A Includes autofluorescence from epidermis and LDE.(or leakage #)
B Omits epidermal autofluorescence. (or measures LDE leakage #)
C Omits dye fluorescence. (signals autofluorescence of media #)
D Net epidermal autofluorescence, A-B. (A-B: Na+ fluorescein #)
E Signal/noise ratio, A-C/B-C.
F Filter sets used in the Cytofluor 2300 with given dye.
G CytoFluor 2300 sensitivity level (gain) used for given dye.

TABLE 3

PRELIMINARY OBSERVATIONS ON FEASIBILITY

No.	Question asked	Current Hypothesis	Basis
1.	Probe/Cytofluor method with LSE?	Signal is readable.	Table 2.
2.	Excessive binding of dyes to LDE, gel?	Dyes bind epidermis 3-12x stripped LSE.	Table 2.
3.	HEM damaged in transit?	Adhesion loss, plasma membrane damage.	Table 1.
4.	Which dyes best show damage?	None excluded, tests with HD required.	Table 1, Table 2.

There are many virtues of fluorochrome probes that make them suitable choices for multiple endpoint assays.[6] First, some can be used to double-label cells.[3] Second, they are non-radioactive. Third, fluorochrome diversity exceeds that of radioactive probes.[5] Fourth, unlike radioactive probes, fluorochromes can be visualized with a fluorescence microscope, or a spectrofluorometer, or both. Fifth, activity/site-specific fluorochrome signals can be analyzed in situ in living cells- a feature not currently possible with radioactive tracers. Sixth, since probes are being created for new purposes,[5] it may soon be possible to label cells with several different probes to reveal concurrent pathobiological responses during a cytotoxic episode. Finally, such testing has promise for saving both time and money by facilitating objective analysis of a variety of responses with a single instrument.

Simultaneous uses of two probes have been reported with both cellular monolayers[3] and HEM.[4] Our data are not yet sufficient to determine that current human skin models will be suitable candidates for a CytoFluor-based, automated, multiple endpoint, fluorescent probe assay of antivesicant compounds. However, existing evidence encourages further efforts to develop such an approach.

CONCLUSION

Incubation of damaged and control human skin models with eight site-specific fluorescent probes resulted in differences of induced or reduced fluorescence as shown by CytoFluor 2300 measurements; the results suggest that these methods may be usable to measure damage from HD.

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THE SOLATEX™-PI SYSTEM AN *IN VITRO* METHOD TO PREDICT PHOTOIRRITATION

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ABSTRACT

A new *in vitro* method, SOLATEX-PI™, has been developed to predict the phototoxic potential of compounds. A phototoxic effect is considered when a significant enhancement of an *in vitro* irritation index is exhibited when the material is excited with radiation in the barrier membrane macromolecular matrix. The SOLATEX-PI System incorporates the direct measurement of acute dermal irritation in a two-compartment physico-chemical model with and without UV radiation to predict potential photoirritation [1,2,3]. The model includes a keratin/collagen barrier membrane, which is compartment one. This is inserted into a biomacromolecular matrix, which is compartment two. UV_A, as well as combined UV_A and UV_B, were used for exposure of the test samples in the SOLATEX-PI System. The test system has been evaluated with 77 materials, which were classified as strong, idiosyncratic and negative photoirritants based on the *in vivo* response. Of 32 photoirritants, 29 were correctly identified. Only two compounds in the borderline group produced significant photo-induced irritation in this system.

Dermal photoirritation is the production of a reversible reaction in the skin after one contact of the skin with a test substance and exposure to UV light. Photoirritation occurs when the dermal irritation response of a test material increases when the system is exposed to a dose of excitating radiation. Photoirritation is produced by all nonimmunologic, light-

induced toxic skin reactions with endogenous or exogenous chemicals which absorb light to an excited state and transfer energy to or react with cellular components. UV light, including combined UV_A and UV_B, as well as UV_A were used to expose samples in contact with the SOLATEX-PI System for varying times and doses. UV_A has elicited most of the known *in vivo* photoirritant responses. In this study, UV_A alone, as well as UV_A and UV_B combined, were used for broad range exposure and to study possible synergistic effects of UV_A and UV_B.

UV radiation consists of light with wavelength from 200-400nm. Radiation with short wavelengths (UV_B) does not penetrate as far into the epidermis in the *in vivo* model. UV_A, of longer wavelengths penetrates further.

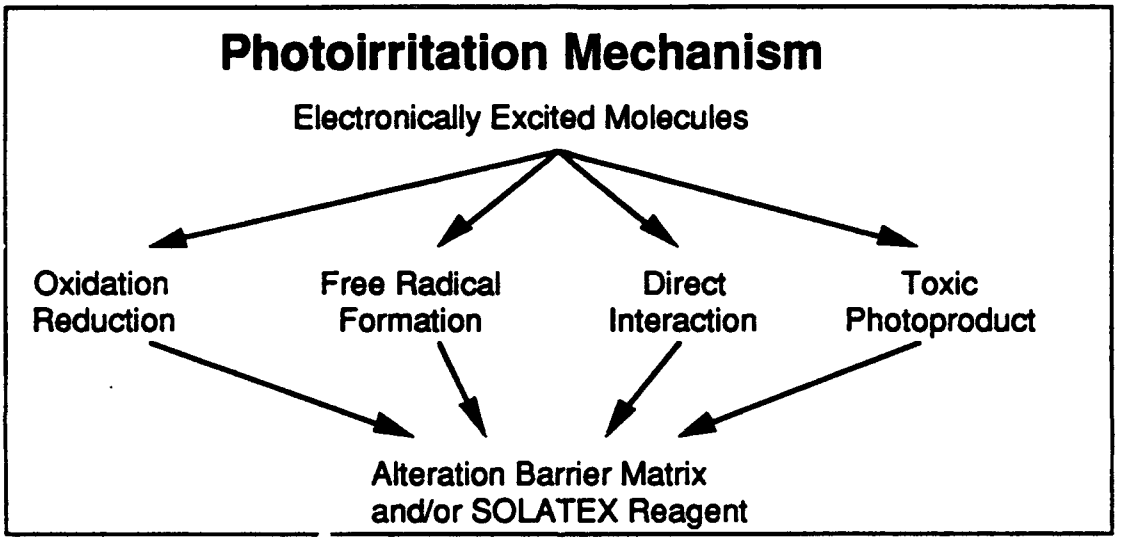
Photoirritation includes many mechanistic pathways [4,5,6,7,8]. The simplest mechanism is the alteration of the test sample to produce a toxic product, which then causes increased irritation. Other pathways require the test sample, the *in vitro* or *in vivo* system, and the UV radiation to interact. Free radicals also form (oxidation reduction products) which then cause alterations in any components of the *in vitro* or *in vivo* system. As chemicals are excited, their excited state can interact directly with components of the *in vitro* or *in vivo* system (see Figure 1).

The basic principle of this method is the quantitation of an enhanced response in a previously described two-compartment physico-chemical model of dermal irritation upon exposure to UV radiation [1,2,3]. Appropriate controls with no UV radiation provide the background irritation response. The irritation response is quantified as the change either in a keratin/collagen barrier membrane or in a highly ordered macromolecular matrix. A red dye attached to the keratin/collagen barrier membrane (compartment one) can be released if the integrity of this barrier is altered. Chemical irritants can perturb the matrix (compartment two) to produce turbidity. The total response, the sum of the response to compartment one and two, can be measured spectrophotometrically.

The major advantage of this model is the rapid, objective and cost-effective methodology. The number of new chemicals introduced yearly is large. They include very diverse structural molecules so all classes of these chemicals could produce adverse photobiological effects. Rapid screening before use would be valuable. *In vitro* testing should include an evaluation of photoreactivity of a new chemical in the presence of biomacromolecules such as SOLATEX-PI, as well as complementary testing in cellular phototest systems.

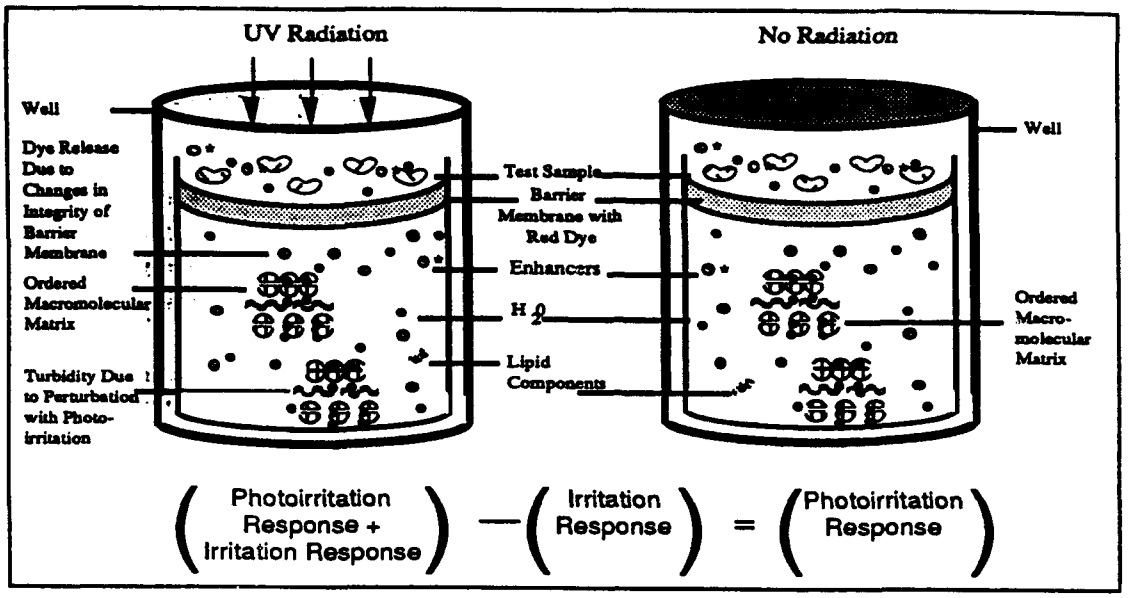
The SOLATEX-PI System permits an evaluation of chemicals and formulations with essential biomembranes and biomacromolecules. The role of biomacromolecular change in adverse photobiological effects is very important. Formation and reaction of intermediates and damage, and reversible and irreversible binding to biomacromolecules provides an indication that adverse light- induced effects can be expected.

FIGURE 1
Photoirritation Mechanism



In the SOLATEX-PI model, when UV radiation alters the test sample or formulation in contact with the two-compartment model, the electronic excitation can produce changes in the barrier membrane or matrix greater than those produced by the test sample in the absence of UV radiation. This enhanced response is the photoirritant response (see Figure 2). The changes can occur by free radicals, toxic photoproducts, oxidation/reduction or direct interaction of the excited chemical with the barrier membrane or reagent matrix.

FIGURE 2
SOLATEX-PI Model



From earlier *in vivo* studies[4,5,6,7,8], different classes of chemicals produce photoirritation via different mechanistic pathways. Target molecules can be proteins, lipids, protein lipid complexes in membrane, ribosomes and DNA (see Figure 3).

FIGURE 3
Mechanism and Targets[4,5,6,7,8]

COMPOUND	MECHANISM	TARGET
Psoralen	Direct Addition Photo Dynamic	DNA Proteins Membranes Riboosomes
Phenothiazine	Toxic Photoproduct	DNA Membranes
Porphyrins	Photo Dynamic	Membranes DNA Proteins
Acridine Orange	Photo Dynamic	Membranes
Anthracene	Photo Dynamic	Membranes DNA

The SOLATEX-PI System uses the principles of the SKINTEX™ test method as a model for *in vitro* dermal irritation. SKINTEX is based on a two-compartment physico-chemical model of dermal irritation which incorporates a membrane barrier and a reagent matrix sensitive to perturbation by chemical irritants [1,2,3]. This is called the Barrier Membrane/ Reagent Matrix System.

The Barrier Membrane/Reagent Matrix has been standardized into a rapid, accurate and reproducible assay. The possible sources of interference in this system were evaluated. The procedure to detect interference was incorporated into the protocol to minimize false negative results.

SOLATEX-PI Barrier Membrane: A buffered salt solution at pH 8.0 of 10% keratin and 1% collagen was bound to cellulose acetate with 0.1% glutaraldehyde at 25°C for one hour. After washing the support in distilled water, Basic Red 2 was attached to the keratin/ collagen matrix with 0.1% glutaraldehyde for 10 minutes at 25°C. Indicator Barrier Matrices were washed exhaustively in distilled water and dried. The matrices were stored at 4°C and have a 180 day shelf life. Circles are cut and formed into a well within a plastic disc.

SOLATEX Reagent Matrix: A lyophilized powder, containing globulins, collagen, glycosaminglycans, free fatty acids, amino acids, phospholipids and buffer salts, was rehydrated. The rehydrated reagent was stable for 15 days at 4°C.

SOLATEX Photoirritant Method: A prototype method was used to establish the relationship between the *in vitro* response for known photoirritants in the presence and absence of UV radiation. Test materials were exposed to UV_A or to UV_A in combination with UV_B. This procedure permitted testing of pure liquids and solids at multiple concentrations. Test samples were applied directly to the barrier membrane, which were then inserted into reagent or diluent filled wells. Test samples, calibrators and controls were incubated at 25°C for 24 hours in the presence and absence of UV light. After incubation the net O.D. at 400nm was determined. Materials which exhibited a greater than 50% increase of their response with UV over their baseline without UV were considered potential photoirritants.

UV Light Sources: The UV light source used for UV_A was a 20W (General Electric F20F12BL) and for UV_B a 20W Sunlamp (Westinghouse FS20). These light sources were housed in a temperature-controlled Solarium developed and designed by INVITRO INTERNATIONAL. In order to maintain constant UV intensity, the irradiance of the UVA and UV_B lights was determined prior to each screen with a radiometer Model ILI400 from

International Light, with either a UV_A or UV_B sensor. In most experiments, the system received a UV_A dose of 0.6-1.2 w/cm^2. The distance of the system from the lights was adjusted to assure these intensities. In experiments utilizing UV_A/UV_B light exposure, the UV_B dose of 0.1-0.5 w/cm^2 was used.

Replicate Analysis: Replicate analysis of the response of background and a known irritant with no UV light demonstrated a baseline of -30% to +30% across a range of baseline irritation of nonirritant to mild irritant. A borderline range from 30 to 50% requires reanalysis of test sample at lower concentrations to qualify the prediction of photoirritation for a test sample.

Range Qualification of Assays: A minimal irritant or solvent must produce a reproducible net change in the SOLATEX-PI System with UV in relationship to the nonirradiated control. A mild irritant must produce within a 30% net increase of the SOLATEX-PI System with UV in relationship to the nonirradiated control. For the entire range of irritancy from nonirritant to mild irritant, uniform changes upon exposure to UV must be observed. A known photoirritant sulfanilamide at concentrations from 1 to 10% must produce an increase of 80 to 260% over background in a qualified assay upon exposure to UV radiation in relationship to the nonirradiated control.

UV Absorption by High Concentrations of Photoirritants: Very high concentrations of potential photoirritants can block UV exposure into the chemical in contact with the skin *in vivo* or in contact with the Barrier Membrane/Reagent Matrix System *in vitro*.

Test Compounds and Formulations: The 65 materials used for evaluation of this assay were obtained from Sigma Chemicals, Aldrich Chemical Company or from test materials submitted to the laboratory by outside laboratories. Pure chemicals, vehicles, mixed vehicles, dilutions, as well as complete mixtures were analyzed.

In Vitro Photoirritant Classes: These are nonphotoirritant, Borderline, Photoirritant, and Strong Photoirritant (see Table 1). These classes correspond to different enhancements (net % increase) of the dermal irritation response.

The compounds chosen to evaluate the *in vitro* photoirritation test were categorized on reported adverse effects in humans [9,10,11,12,13,14,15,16,17] (see Table 1). Guinea pigs, minipigs, hairless mice, rats and rabbits are generally used in tests for phototoxics. Often *in vivo* responses in these other species are quantitatively and qualitatively different from the comparable responses in humans (see Table 1). Many photosensitizers may be phototoxic if an endpoint different than erythema is used as an indicator of phototoxicity [18].

TABLE 1
***In Vivo* Database**

Chemical	Conc.	LightSource	Photoirritant	Reference
Acetone		UVA	Non PI	20
Acetophenone		UVA	PI	21
Acid, 3 Nitro Benzoic		UVA	PI	22
Acid, Acetylsalicylic		UVA	Non PI	22
Acid, Anisic		UVA	PI	23
Acid, Kynurenic		UVA	PI	22
Acridine Orange		UVA	PI	24
Amiodarone		UVA	PI	41
Anthracene		UVA	PI	14
Benzaldehyde	1%	UVA	PI, PS	22
Benzoic Acid	5%	UVA	I	26
Benzophenone	1%	UVA	PS	21
Bergamot Oil		UVA	PI	38
Bithionol	1%	UVA	PI, PS	27
Chloride, Benzalkonium	0.10%	UVA	Non PI	26
Chloroquine		UVA	PI	28
Chlorpromazine	1%	UVA	PI, PS	29
Chlorpromazine	.1%	UVA	PS	29
Chlorpromazine		UVA	PS	29
Coke, Heavey	10%	UVA	PI	20
Cold Cream	100%	UVA	Non PI	20
Coumarin		UVA	Non PI	22
Coumarin, 6-Methyl	1%	UVA	PS, PI	30
Coumarin, 7-Methyl		UVA	PI, PS	30
5 Chlorosalicylanilide	1%	UVA	PI	30
Dimethylchlortetracycline		UVA	PI	39
Dinitrochlorobenzene		UVA	Non PI	26
Doxycycline		UVA	PI	37, 39
Eosin Y		UVA	PI	31
Ethylene Glycol	100%	UVA	Non PI	20
ETOH/PPG	100%	UVA	Non PI	20
Fluoranthene		UVA	PI	14
Fluorene	1%	UVA	PI	14
Glycerol	100%	UVA	Non PI	20
Halogenated Salicylanilides		UVA	PS	37
Household, Black Liq.	10%	UVA	Non PI	20
Hydrochlorthiazide	1%	UVA	PI	34
Hydrocortisone		UVA	Non PI	26
Isopropyl Palmitate	1%	UVA	Non PI	20

TABLE 1
***In Vivo* Database**

Chemical	Conc.	LightSource	Photoirritant	Reference
Isopropylamine	1%	UVA	Non PI	20
Methylene Blue	1%	UVA	Non PI	3
Mineral Oil	100%	UVA	Non Pl	20
Moisturizer	100%	UVA	Non PI	20
NaCl	0.90%	UVA	Non PI	20
Nalidixic Acid		UVA	PI	23
Oil, Crude	10%	UVA	Non PI	20
Oil, Slurry	10%	UVA	Non PI	20
PABA		UVA	PS	33
Phenanthrene		UVA	PI	14
Phenergan		UVA	PI	40
Phenothiazine		UVA	PI	34
Piroxicam		UVA	PI	14
PPG	100%	UVA	Non PI	20
Promethiazine		UVA	PI	40
Psoralen, 5 Methoxy	1%	UVA	PI	35
Psoralen, 8 Methoxy	1%	UVA	PI	35
Pyrene		UVA	PI	14
Quinidine		UVA	PI, PS	36
Salicylamine		UVA	Non PI	30
Salicylic Acid		UVA	Non PI	26
Skin Lotion	100%	UVA	Non PI	20
Sulfanilamide	1%	UVA	PI	34
TCSA		UVA	PI, PS	
Tetracycline		UVA	PI	37
Thiourea		UVA	PI	22
Triton-X	10%	UVA	Non PI	30
Tween 80	10%	UVA	Non PI	30
Water	100%	UVA	Non PI	26

Test samples were prescreened to determine a level of the test material or formulation that did not cause moderate or severe irritation. At least two different concentrations were tested. Photoirritant studies *in vivo* also must be performed at concentrations which produce a minimal level of dermal irritation. This permits the best evaluation of an enhanced dermal irritation response upon exposure to UV radiation (see Table 2).

TABLE 2
***In Vitro* Photoirritant Classes**

NET % INCREASE	*IN VITRO* CLASS
<30%	Non PI
30-50%	Border
51-100%	PI
>101%	Strong PI

The use of UV_A, or UV_B, with UV_A was evaluated in these studies. Recent studies investigating photosensitization have used UV_A and UV_B for broad range screening [26]. The presence of UV_A and UV_B can be made more sensitive and UV_B may cause changes which deactivate or alter a molecule electronically excited by UV_A [42, 43].

Studies were performed with UV_A, or a combination of UV_A and UV_B. When the known photoirritant sulfanilamide at 1.5% was analyzed, UV_B alone demonstrated the largest net % increase at 16 hours. At 24 hours, UV_A and UV_B demonstrated a similar increase. At 24 hours of UV_A alone, about 66% of this increase was observed (see Figure 4). Similar results were obtained in a study of sulfanilamide at 5%. At eight hours with UV_B alone, the largest net percent increase was observed for 5% sulfanilamide (see Figure 5).

Results were summarized at two to 10 concentrations. Only results from qualified assays where the background control and irritant control produced minimal changes upon exposure were evaluated. The known *in vivo* photoirritant had to produce a PI response for the assay to qualify also. The qualification of an assay is summarized in Figure 6, with the dose response curve for 3- nitrobenzoic acid.

A complete presentation of results is summarized in Table 3, where the relevant *in vivo* results from Table 1 are included for comparison.

FIGURE 4

Net % Increase Produced by UV_A (●),UV_B (□) and $UV_A + UV_B$ (⊣●⊢) with 1.5% Sulfanilamide in SOLATEX-PI

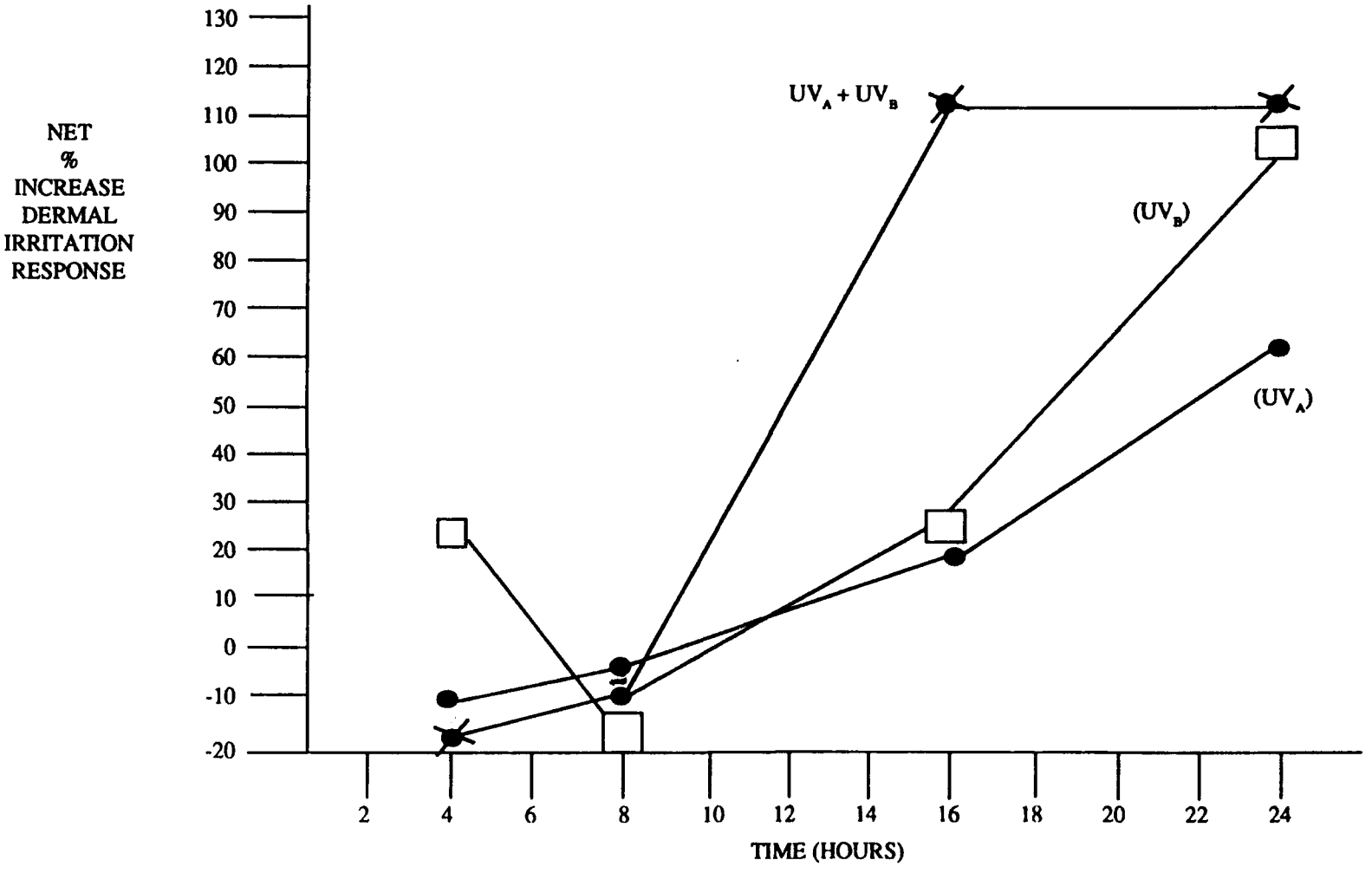


FIGURE 5

Net % Increase Produced by UV_A (●), UV_B (□). and $UV_A + UV_B$ (✕) with 5% Sulfanilamide in SOLATEX-PI

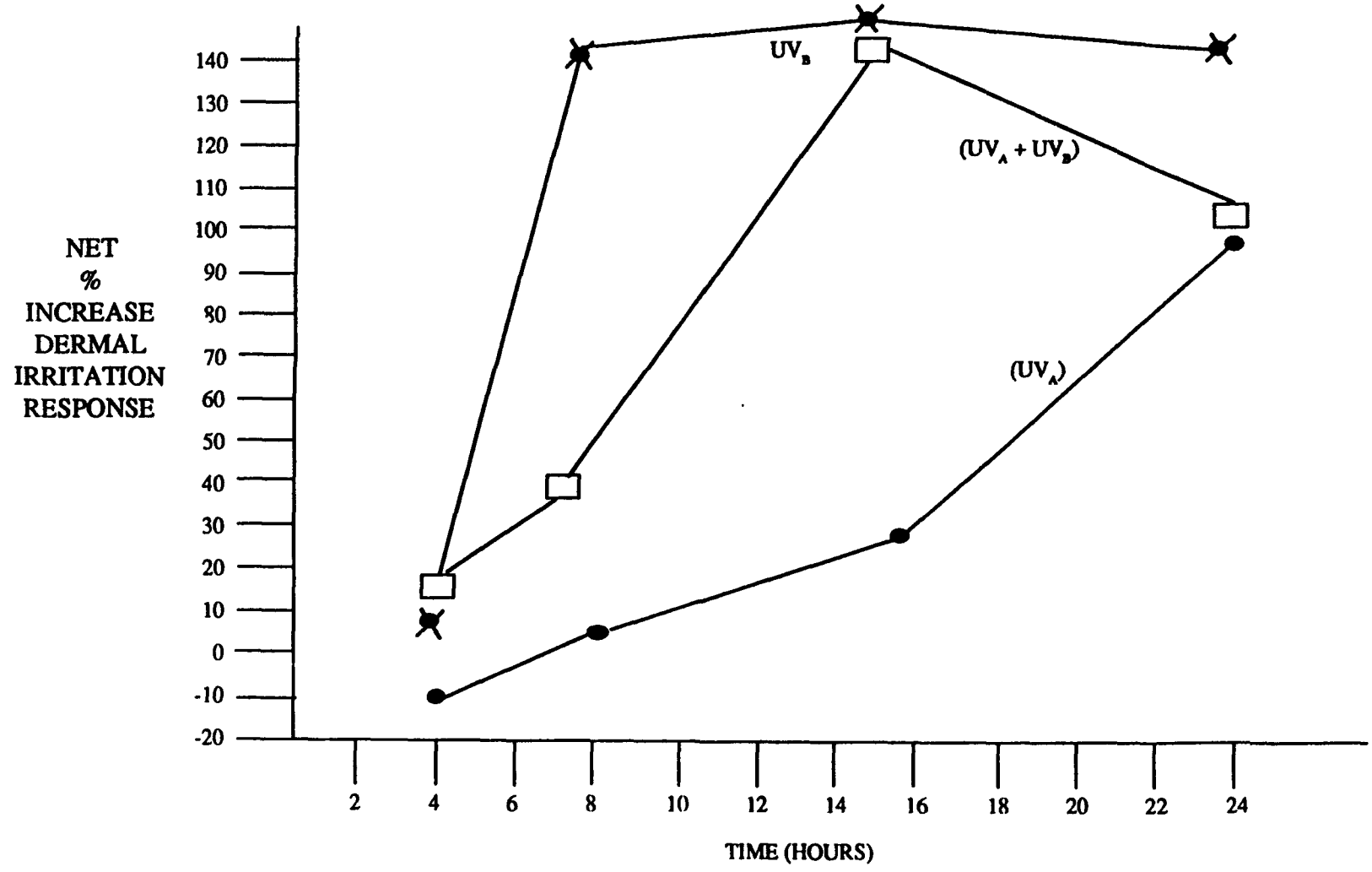


FIGURE 6
Photoirritant Response of Acenapthene

Sample	:	Acenapthene
Class	:	Coal Tar
Analysis date	:	08/09/91
NTC #	:	DFU32
Protocol	:	SOLATEX Pi UVA-UVB
User name	:	Kathryn
Sample classification	:	PHOTOIRRITANT (3.0%)

Name	UV OD	No UV OD	Samp %	Net %	Qualification
SX0	480	258	.86		Qualified
SXI	932	520	79	-7	Qualified
PI 1	780	271	188	102	Qualified
PI 5	880	256	244	158	Qualified
PI 8	795	259	207	121	Qualified
PI 10	941	308	206	120	Qualified
Solvent	549	298	84		Qualified
Sample	651	253	157	73	Qualified
Sample	1044	394	165	81	Qualified

SOLATEX PI DOSE RESPONSE

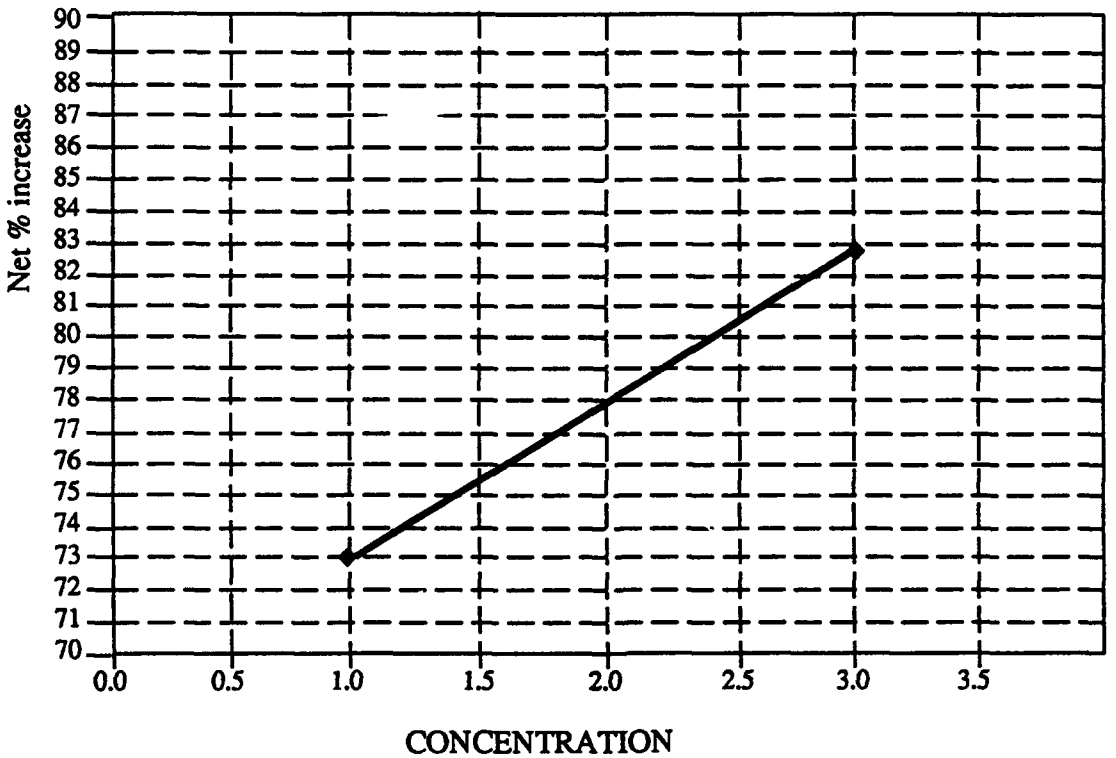


TABLE 3
Summary of *In Vitro* and *In Vivo* Results

Test Sample	UVA ONLY Class *In Vitro*	UVA ONLY *In Vivo* Response
Acetone	Non PI	Non PI
Acetophenone	PI	PI, PS
Acid, 3-Nitro Benzoic	Strong PI	PI
Acid, Acetyl Salicylic	Non Strong PI	PI
Acid, Anisic	Strong PI	PI
Acid, Benzoic	Non PI	Non PI
Acid, Salicylic	Strong PI	Non PI
Acridine	Strong PI	PI
Anthracene	Strong PI	PI
Benzalkonuim Chloride	Non PI	Non PI
Benzaldehyde	Non PI	PS
Benzophenone	Non PI	PS
Bergamot Oil	Non PI	PI
Bithional	PI	PI, PS
Chloroquine	Non PI	PI
Chlorosalicylanilide	PI	PI
Chlorpromazine	Strong PI	PI, PS
Cinnamyl Alcohol	Non PI	Non PI
Coke, Heavy	Strong PI	PI
Conditioner #2	Non PI	Non PI
Coumarin, 6-Methyl	Strong PI	PI, PS
Coumarin, 7-Methyl	PI	PI, PS
Cream Deodorant	Non PI	Non PI
Crude Oil	Non PI	Non PI
Doxycycline	Strong PI	PI
Eosin Y	PI	PI
ETOH/PPG	Non PI	Non PI
Eythlene Glycol	Non PI	Non PI
Ethylhexyl Methoxy Cinnamate	Non PI	Non PI
Fluorene	Strong PI	PI
Glycerol	Non PI	Non PI
Heavy Coker Gas Oil	PI	PI
Hexachlorophene	Non PI	Non PS
Household Black Liq.	Non PI	Non PI
Hydrochlorthiazide	PI	PI
Insecticide	Non PI	Non PI
Isopropyl Palmitate	Non PI	Non PI
Isopropylamine	Non PI	Non PI

TABLE 3
Summary of *In Vitro* and *In Vivo* Results

Test Sample	UVA ONLY Class *In Vitro*	UVA ONLY *In Vivo* Response
Methylene Blue	Non PI	Non PI
Mineral Oil	Non PI	Non PI
Moisture Balm	Non PI	Non PI
Moisture Base	Non PI	Non PI
Nalidixic Acid	PI	PI
Octyl Salicylate	Non PI	Non PI
Oil Slurry	Non PI	Non PI
PABA	Non PI	Non PI
Phenanthrene	PI	PI
Phenothiazene	PI	PI
Phenylbutazone	PI	PI
Piroxicam	PI	PI
PPG	Non PI	Non PI
PG	Non PI	Non PI
Psoralen, 5-Methoxy	Non PI	PI
Psoralen, 8-Methoxy	Non PI	PI
Quinidine	PI	PI
Retinoic Acid	Non PI	Non PI
Salicylamide	Non PI	Non PI
Salicylanilide	Non PI	Non PI
Sulfanilamide	Strong PI	PI, PS
TCSA	PI	PI, PS
Tetracycline	Strong PI	PI
Triton X 100	NQ	Non PI
Tween 80	Non PI	Non PI
Water	Non PI	Non PI

CONCLUSION

In this paper, the use of a quantifiable *in vitro* dermal irritation model for photoirritancy testing is presented. The *in vitro* method described provides a simple and rapid method to screen for photoirritants. Photoirritants, which act on different cellular sites of action, produce significant increases in dermal irritation *in vitro* on exposure to UV light in SOLATEX-PI. The results show the group of strong photoirritants produced increases in the dermal irritation response of 100 to 300%. Their potential photoirritancy was correctly indicated in the *in vitro* test. Test samples in the idiosyncratic and negative group of *in vivo* produced little effects. A low number of false positives is beneficial for photoirritation screening.

Many test materials, which are known photoirritants, are not detected in the yeast phototoxicity assay. These materials manifest a persistent erythema rather than the hyperpigmentation produced by tars and furocoumarin readily detected in yeast phototoxicity assay. Photoirritant test materials observed *in vivo*, which produce response in SOLATEX-PI and not in the photo yeast assay, are sulfanilamide and acetylsalicylic acid and dimethylchlortetracycline [25,44]. The microbial test system may correlate with one of several types of photoirritation. Phototoxicity studies with cells do not demonstrate sensitivity to chlorpromazine and tetracycline and sulfanilamide [46,47]. The psoralens are readily demonstrated in yeast phototoxicity assay which has the capability to predict mechanisms which involve DNA changes, but are not demonstrated in SOLATEX-PI. The coumarins are readily discerned in both systems [25].

In order to look at the complete UV spectrum, it is important to perform studies with UV_B as well as UV_A light [26]. Due to the lethal of effect UV_B, yeast phototoxicity tests can only be performed with radiation >320 nm. SOLATEX-PI can incorporate evaluation throughout the UV spectrum.

No one test system has the complete ability to predict the photoirritant potential of chemicals, fragrance oils, natural products, formulations and sunscreens [45,46]. Some of these discrepancies are due to variations of absorption, enzyme-induced transformation, as well as cutaneous or metabolic inactivation. The SOLATEX-PI System demonstrated the capability to predict many photoirritants other than the psoralens. This model is a useful screen for predictive photoirritant testing. The major advantage of this model is the rapid, objective and cost-effective methodology. The number of new chemicals which are introduced yearly is large. They must include an evaluation of photoreactivity of a new chemical in the presence of biomacromolecules, such as SOLATEX-PI, as well as complementary testing in cellular phototest systems.

The SOLATEX-PI system permits an evaluation of photoreactivity of chemicals and formulations with essential biomembranes and biomacromolecules. The role of biomacromolecular change in adverse photobiological effects is very important. Formation and reaction of intermediates and damage, and reversible and irreversible binding to biomacromolecules provide an indication that adverse light-induced effects can be expected. With this evidence of photobiological effects, an evaluation can be continued with bacterial or cell systems. These methods can be integrated as part of a protocol for *in vitro* testing of the photoirritant potential of chemicals.

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SUMMATION-WRAP-UP

ALAN M. GOLDBERG, PH.D
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I'd like to personally acknowledge Harry's contribution for organizing, structuring and arranging this conference. It has been a remarkable program.

Let me share some of my thoughts about this meeting and in vitro toxicology in general. The number of papers that have appeared dealing with in vitro science are markedly increasing. This can be quantified from the National Library of Medicine references on in vitro methodology which I believe is now in its fifth year of publication. This year I believe there are about 33 or 34 pages of publications on in vitro methodologies. Only 5 years ago it was just a page or two. We are seeing a number of companies that never did in vitro now doing in vitro, and those who've been doing in vitro increasing their activities. We are seeing the beginning of an industry devoted specifically to in vitro sciences. Companies who are not doing in vitro themselves, are contracting to have it done for them. We have journals devoted to in vitro, and it's not just one or two, but four or five. One of the previous speakers talked about the first Conference on Teratogenesis which was devoted to in vitro; the date on the slide was 1982. We are looking at a ten year history of activities that is remarkable.

If I can take a commercial break, the next Symposium dedicated to in vitro will be held in Baltimore, April 14-16, 1992. I point this out for two reasons. First, the night

before the conference, there will be a series of commercial presentations available to all. Secondly; the regulatory scientists, we heard Dick Hill talk about the IRAG group, are organizing a meeting to be held in conjunction with the conference to set the agenda for a program on alternatives to the Draize Eye Test. If you are interested, the planning meeting will be held on April 15.

A good talk, I was told many years ago, is one that tells them what you are going to tell them, tells them, and then tells them what you told them. I'm only going to tell you what you've already heard.

In Harry's introduction, he pointed out that many of the major, major advances that have occurred could not have occurred without the use of animals. Every one of Harry's examples were absolutely correct, However, I must add that was only part of the story. What was missed is that these advances could not have occurred without the use of in vitro methods as well. It's not only animals, and not only in vitro, it's a combination of both. It's important to make this point over and over again, because that is reality. Harry, you stated that this conference was designed to highlight new approaches and concepts and to review the advances made for alternatives to animal testing. From what I heard at this conference and your criteria, this conference has been a remarkable success.

This conference demonstrated that we have made progress in in vitro sciences. I'm

always asked by the media and press, and sometimes in a hostile way, "have we really reduced animal use?" We can tell them about pregnancy testing and point out that at one time it was animal based, and its now exclusively in vitro. They say that's only one example. Then we say, how about pyrogen testing. We used to use rabbits for measuring bacterial endotoxin, now we use the limulus lysate assay for this measurement. They say that was an easy one to get rid of. Give me some real examples. Well, how about all the hormone assays that were bioassay and are now being done by HPLC. They say, "but that's just a chemical assay". The point is that we have significant examples of significant progress in this field over the span of our ten year history, more than we could have hoped for.

Charles Tyson pointed out that at the Society of Toxicology Meetings twenty five percent of the papers are now devoted to in vitro studies. Gerry Zbinden did much the same kind of calculation for the FASEB Meeting and found that in 1988 or 1989, fifty percent of the papers were in vitro and fifty percent in vivo. In vitro is not something that is adjunct or supplemental, it is coincident and important. Procter and Gamble has published that over the last five years, they have reduced animals in household and consumer goods testing and development by eighty-nine (89) percent. An eighty-nine (89) percent decrease in terms of their animal use. The fact that the regulatory agencies in Europe and the U.S. have all begun to harmonize the requirements for toxicity testing, is yet another example of where we're going. We have made incredible progress. Absolutely incredible progress. However, I don't think

that we have yet begun the growth phase of the exponential curve. I think that is yet to happen.

I'm not going to summarize each of the presentations, but will try to highlight points made by several speakers that exemplify some areas of my interest. Mel Anderson, among others, called for the increased use of human tissue for in vitro studies. As a member of the board of a company that actually sells human cells, I agree (Note added in proof, I am no longer a member of this board as I resigned May, 1992). However, and more important, the reason we talk about human cells is the possibility that we can decrease the uncertainty introduced by extrapolation. It's not that we know the answer, it is, however, testable and that's the important part. We'll know if human cells offer unique opportunities.

Howard Maibach has stated, "that skin is not skin is not skin". He showed that there are differences in sensitivities from the wrist to the elbow. This changing sensitivity can be shown for skin all over the body. Skin is not skin is not skin. If this is true, and I believe it is, how can we ever hope or look for a single or simple assay. I'm having trouble selecting the right word. Both words are probably correct. There are no simple assays. There are going to be complex assays and many of them. You've heard me say before that what we need are mechanistically-based tests.

Correlative tests may have a place, but I believe it's a very limited place. Correlative

tests only allow us to predict the chemicals that were in the battery that allowed us to establish the test. The weekly news magazine, *The Economist*, once indicated that correlative tests lead to trivial understandings. The example they used was on the noise associated with the firing of a gun. There is no relevance of that noise to the functioning of the gun. This may be true for some of the current correlative tests.

The fact is that we are at the beginning. We have heard beautiful and elegant presentations at this conference. Some of these may lead to more mechanistically-based tests. One of the things we hear when we talk about mechanistically-based tests is that we are going to need lots and lots of tests. There may be hundred and thousands of tests. For some of us, that's a depressing thought, but, I see it as a unique opportunity. We can really make a difference.

We heard our colleagues from the regulatory sector share with us the role that they are beginning to take and pointed out the major advances and refinement to date in the Draize Eye Test. Some non-regulatory participants very quickly pointed out that we shouldn't forget the needs for chronic eye irritation tests. That's a well placed comment. What it indicates is that all of the communities that are involved are concerned that the tests that we use adequately protect the health of the public, and that is all of our missions.

I pointed out that progress has been made. The critics and cynics among us, and I

quote Sheldon Kopp from his escatological list, states, "progress is an illusion". Not true in this area.

The issue of validation has come up many times and it's misunderstood. I know that when I started out in this field, validation was to me something that just happened. Clearly this is not the case. In a recent novel by John Kenneth Galbraith called *A Tenured Professor*, he describes that tenure may actually destroy what it is meant to protect. Instead of protecting dissenting opinion, it may restrain early outspokenness of faculty. I hope that the definitions and restraints that we put on validation, especially on some of the earlier, inadequately developed methods, do not destroy that which we all hope to accomplish.

One speaker stated that in vitro methods must give the same information as an in vivo test. I don't think this is fully correct. I would hope that replacement methods would do better and provide more specific information, and we have examples that this has already occured. In vitro methods are much more specific. It's difficult to compare a hormone assay in an animal with the quality of information of a HPLC printout. The HPLC is clearly a better set of information. However, I will also agree with the speaker because he pointed out the absolute essential necessity for mechanistically-based tests in the regulatory process.

Advances have been described throughout this conference. I would like to end,

however, with a cautionary note. In the book, *Zen and the Art of Motorcycle Maintaince,* Robert Prisic describes the assembly of a barbecue. For those of you who read it and remember it, there's a very vivid description of putting a barbecue together. He points out that at no point do we deviate from taking part A to part B. number 1 to number 2 to number 3. We are stuck in a very rigid structure of how we function. We have to be very careful that we don't get into a very rigid structure and limit our thinking. We must allow flexibility, and continue to develop mechanistically-based tests. From what I heard at this conference, I am very encouraged that we're doing just that. Thank you all very much.

ALAN M. GOLDBERG, Ph.D.

Dr. Goldberg is Associate Dean for Research, The Johns Hopkins School of Hygiene and Public Health, Professor, Environmental Health Sciences, and Director, The Johns Hopkins Center for Alternatives to Animal Testing (CAAT). Recent appointments include Principal Research Scientist, Chesapeake Bay Institute; Director, Division of Toxicology, Department of Environmental Health Sciences, The Johns Hopkins School of Hygiene and Public Health; Director, The Johns Hopkins Associates in Environmental Health; Director, Industrial Liaison Office, The Johns Hopkins School of Hygiene and Public Health;; Visiting Professor, Hadassah Hospital, Jerusalem, Israel.

Dr. Goldberg's educational background includes Polytechnic Institute of Brooklyn, 1956-57, Queens College, 1958, Brooklyn College of Pharmacy (B.A.), 1957-61, University of Minnesota (Ph.D.), 1961-62 - Pharmacology under F.E. Shideman.

Professional memberships include American Society of Pharmacology and Experimental Therapeutics, American Society of Neurochemistry, Society for Neurosciences, Society of Toxicology, and American College of Toxicology. Community activities include Rotterdam Sister City Committee, The Governor's Task Force of Animal Issues, Maryland Council on the Scientific Use of Animals. Honorary Societies, Awards and Honors: Indiana Neurological Society Award, Rho Chi Society, Sigma Xi, Delta Omega, and Who's Who in America.

Dr. Goldberg is also the author/co-author of over 130 publications.

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CONCLUDING REMARKS

Harry Salem

Thank you Alan for those kind words, serving on the Steering Committee and putting the last few days into perspective, and giving us a glimpse of the future.

As chairman of this symposium, I would like to express my sincere thanks and appreciation to all of those who contributed and participated to make this symposium a success.

To all of those who served on the Steering Committee, the Local Committee, the Co-chairs, the platform and poster presenters, as well as to the attendees, I express my thanks, and especially to Mel Anderson for his keynote address and to George Koelle for his delightful after dinner talk on "Poisons of the Cannon."

In addition, special thanks are also due to the people in the Visual Information Division as well as to Lisa McCormick of Science and Technology Corporation for coordinating this meeting and especially to my secretary, Jean Polidore, for all her help and understanding during this process. I guess that the success of a meeting can be measured by the chaos behind the scenes, which remains there, and does not become visible.

I am really delighted and gratified at the overwhelming response we've had to this symposium. On a comparative basis, the first announcement went out rather late, with the 25 January deadline for reservations. By that date we had received one hundred and ninety three pre-registrations. On Monday February 3rd, the day before the symposium, there were two hundred and ninety six pre-registrations, which far exceeded the 217 seating capacity of the Conference Center. However, due to the foresight and cooperation of the Visual Information Division, the program was televised live into the Seminar Area for the overflow crowd. Of the two hundred and sixty one attendees, there were representatives from most of the military services, other government agencies, industry and academia. In addition to the United States, there were representatives from Canada, Israel and the Netherlands. I welcome you all and thank you for coming and participating.

As Alan pointed out in his summation, in the ten year history of alternatives, the in vitro techniques that people have been using are being put into perspective, and real programs are being formulated. Of course, we don't want to do this at the expense of human safety. I think as committed and dedicated scientists, we have a heavy task ahead of us; we're not there yet, even though, as Alan pointed out, a lot of progress has been made. Those of us working in the field, and those of us in attendance and participating appear to have accepted the challenge to continue the pursuit of alternatives. I agree with Alan that the advances made in medicine required a combination of both in vivo and in vitro studies. In order to continue to make progress we still need a combination of both in vitro and in vivo animal studies.

for those of you who are interested in recieving the Bibliography on Alternative methodology that Alan referred to, it can be obtained from the Toxicology Information Program, National Library of Medicine, Bethesda, Maryland 20894, or if you Call Art Wykes at (301) 496-5022, he has graciously agreed to add your name to their mailing list. Thank you Art.

All of us together have taken an important step in accomplishing the three R's. Replacement of animals where scientifically feasible, Refinement of current techniques so we can get the maximal information from fewer animals, and thus Reduce the number of animals, without compromising public health and safety. In addition, I think that we have also shown a commitment to the fourth R, Responsibility.

As committed and dedicated scientists, we have a heavy task and great responsibility in attempting to achieve these goals. As the eyes of all segments of society are upon us, it appears that because of well attended symposia such as this, and the research activity in the quest for alternatives, we have accepted the challenge.

I hope that this may be the spearhead for continued activity in this worthwhile endeavor, and in particular to mobilize the knowledge being generated in molecular biology and genetic engineering to accomplish our mission of the three R's, and continue a commitment to the fourth R, Responsibility. Thank you again for your attendance and participation.

ANNEX A

Index of Authors

PROCEEDINGS OF THE SYMPOSIUM
ON CURRENT CONCEPTS AND
APPROACHES ON ANIMAL TEST ALTERNATIVES

APPENDIX

LIST OF ATTENDEES

Attendee	Agency/Company	Telephone
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